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AN ADAPTATION OF WINKLER'S METHOD TO BIOLOGICAL WORK.

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Winkler's method for the determination of oxygen in solutions has been extensively employed in biology. The chief obstacles to a successful application of the method are:

1. The necessity of removing the organisms from the solution before adding the reagents. In doing this oxygen may be admitted in spite of all precautions. If the other alternative be adopted, namely, siphoning off a sample of the solution, the danger of contamination with oxygen still exists.

2. The desirability of taking samples from time to time in order to follow the course of the reaction by which oxygen is produced (as in photosynthesis) or consumed (as in respiration).

In order to obviate these difficulties the writers have employed the apparatus shown in Fig. 1. *A*. It consists of two Pyrex (or "Nonsol") glass tubes *G* and *H* (each about 30 cm. long and of about 27 mm. diameter) joined by a piece of flexible rubber tubing *F* (all the rubber tubing and rubber stoppers used in the apparatus should be repeatedly boiled and coated with paraffin) and furnished with rubber stoppers at the free ends.

One of the rubber stoppers is connected with a series of smaller glass tubes (*B*, *C*, *D*, *E*) of not less than 13 mm. inside diameter, with only enough space between them to permit the insertion of a clamp.

The procedure is as follows. The larger tubes are filled with the solution containing the organisms by pouring through *E*; or, if the organisms are too large to pass through *E*, they may be placed in the large tubes before they are joined by the flexible rubber tubing *F*. In the case of marine algæ the material may be so large that when once placed in the tube it cannot easily be

moved (by inverting or jarring the apparatus). In such case care should be taken to place the material in the large tube *G* so that when the clamp *K* is applied at *F* none of the material protrudes into *H*.

The amount of solution placed in the apparatus is carefully measured. The liquid is poured in until the level reaches the clamp between *B* and *C*. Small glass beads covered with paraffin may be placed in the solution to act as stirrers.

After closing the clamp between *B* and *C* (avoiding the inclusion of air) the apparatus is left until the determination of oxygen is made. This is done in the following manner. The apparatus is inverted several times in order to mix the contents thoroughly. This may be facilitated by attaching the entire apparatus to a piece of board. The organisms are collected in *G* (by gravity, assisted by shaking, or by means of a device to be described later); *G* and *H* are pulled slightly apart, causing the rubber tube *F* to collapse slightly. The clamp *K* is then used to close *F* completely (the jaws of the clamp are prevented from cutting *F* by strips of soft leather). The tube *G* is then removed and the volume of solution in it is measured.

The clamps above *B* are opened and alkaline potassium iodide[1] is poured into *C* until the level appears in *D*. The clamp between *C* and *D* is then closed; the excess of reagent is poured off and the tubes *D* and *E* are thoroughly rinsed. The solution of manganese chloride is poured into *D* until the level is seen in *E*. The clamp between *D* and *E* is then closed; the excess of reagent is poured off and the tube is thoroughly rinsed. (The tube *D* is about as long as *C*.) The clamp between *B* and *C* is then opened; the apparatus is inverted several times to mix the reagent with the solution in *H*.

The tube *E* (which is about three times as long as *D*) is filled with concentrated hydrochloric acid, and clamped off (so as to include no air); the excess of reagent is poured off and the tube rinsed. The clamp at the free end of *E* should be closed with special care to prevent the sucking in of air.

The clamp between *C* and *D* is now opened and the apparatus

[1] The amount will depend on the strength of the reagent and the quantity of oxygen present, and must be regulated by varying the size of *C* and the length of the rubber connection.

inverted several times to mix the reagents (the rubber connections should be squeezed, if necessary). When the mixing is finished the clamp between *D* and *E* is opened and the apparatus allowed to stand (*E* being above) without inverting (squeezing the rubber connections if necessary). The acid falls and mixes

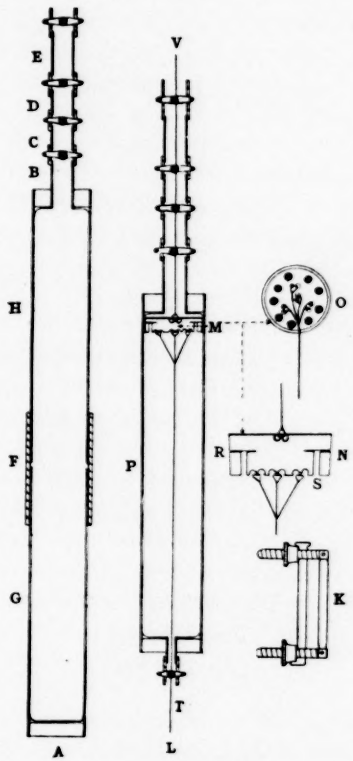

FIG. 1. Apparatus for determining the amount of oxygen in solution by Winkler's method.

by its own weight, dissolving the precipitate in the tube. When this is accomplished the clamp at the free end of *E* is opened and the liquid is poured out into a beaker. The tube *H* is rinsed with distilled water which is then poured into the beaker. Titration is then performed in the usual manner. The volume of solution

is found by subtracting the volume in *G* from the total amount placed in the apparatus at the start.

The writers have found it advisable in most cases to use 0.01 N sodium thiosulfate (containing 4 cc. of 1 M sodium hydroxide per liter) in place of 0.1 N as usually recommended.[2]

A control is made in exactly the same manner except that the organisms are absent.

The essential feature of this apparatus is the provision for collecting all the organisms into one of the large tubes while the oxygen in the other tube is determined. In some cases the organisms cannot be collected into one tube by gravity (assisted by shaking). In this case the apparatus shown in Fig. 1, *L*, is used. The essential feature is a piston *M* to which are attached strings, of small diameter but sufficiently strong, by which it can be pulled through the tube *P*, sweeping the organisms with it, so as to collect them in one end of the tube.

As shown in Fig. 1, *N*, the piston consists of a rubber stopper (snugly fitting the tube *P*). A circular cut *R* is made parallel to the plane surface of the stopper and holes *S*, *S* are bored as far as the cut. When the stopper is pulled through the tube in the direction of the letter *S* in the figure, the solution passes through the holes *S*, *S*, passes out through the cut (which opens under the pressure), and slips between the piston and the glass. The ease with which the liquid slips past the piston depends on the width of the flap and the taper of the stopper. If necessary bolting cloth may be placed over the holes *S*, *S* to strain out very small organisms. The number of holes may be varied. Fig. 1, *O*, shows a convenient arrangement (the holes are shown in solid black).

The strings may be attached in any convenient manner. The arrangement shown in Fig. 1, *O* and *N*, is obtained by boring holes in pairs to a moderate depth and puncturing the connection between each pair so as to admit the string.

When the apparatus is set up the plunger *M* is placed as shown in the figure. Solution and glass beads are placed on both sides

[2] Treadwell, F. P., Analytical Chemistry, translated by Hall, W. T., New York, 4th edition, 1915, ii, 760. For directions for making the dilute solutions see Birge, E. A., and Juday, C., *Wisconsin Geol. and Nat. Hist. Survey, Series 7, Bull. 22*, 1911, 13–21.

of *M* and the clamps closed. When the oxygen is to be determined the organisms are swept out of the tube by pulling the plunger as far as possible toward *T* (without opening the clamp). The plunger is then pulled back (toward *V*) just enough to straighten the flap of the stopper so that it fits the tube snugly and prevents the passage of liquid.

In a special case it might be possible to place the tube on a centrifuge (the end at *T* being fused instead of closed by a stopper). The organisms would then be collected by centrifugal force at the end *T* and the piston would automatically follow them.

The solution, freed of organisms, is treated as previously described. The volume is easily ascertained by pouring in liquid through the end (at *V*) until it reaches from the piston (in its final position) to the first clamp.

It is of course desirable to invert the apparatus frequently during the experiment so as to mix the contents of the tube. (The first small tube above *M* need not be large enough to introduce an appreciable error due to the fact that the liquid in it does not come into contact with the organism. This error is, in any case, a constant one.)

In some cases it is desirable to follow changes in the reaction of the solution. For this purpose an indicator may be added to the solution at the start. This must be such as not to injure the organisms or interfere with the determination of oxygen (phenolphthalein may be recommended for this purpose). The color of the solution may then be observed at any time (preferably through the small glass tube *B*) and compared with buffer solutions (of known hydrogen ion concentration) contained in tubes of the same diameter and containing indicator of the same concentration.[3]

In order to take samples during the progress of an experiment the tubes *G* and *H* are replaced by large T-tubes which are connected end to end (several may be placed in series, attached to a board, so as to be easily handled). A set of small tubes (*B*, *C*, *D*, *E*) is attached to each T-tube by its side arm. Whenever it is desired to make a determination the T-tube at the end is

[3] Haas, A. R., *Science*, 1916, xliv, 105.

clamped off (after clearing it of organisms) and the usual procedure is followed.

Allowance must be made for the fact that after any T-tube is clamped off the organisms are in a smaller volume of liquid. As the oxygen content is known this presents no difficulty.

The simplest method of following a reaction is to renew the solution completely after each determination is made. If for example the effect of an anesthetic on oxygen consumption is to be determined a considerable quantity of the solution containing the anesthetic is made up at the start. Some of this is placed in the apparatus shown in Fig. 1, *A*. When the tube *H* is clamped off at *F* the organisms in *G* are rinsed in a fresh lot of the solution either by allowing them to settle and then decanting or by pushing down the piston *M* into the tube, pouring out the liquid, adding fresh solution, and pulling out the piston. The tube *H* is then replaced by a similar tube, which is joined to *F*. The whole apparatus is filled with fresh solution and the usual procedure followed.

In this way the supply of oxygen is kept up by frequent renewal and determinations may be made as frequently as desired.

SUMMARY.

The adaptation of Winkler's method here described permits:

1. Removal of the organisms before adding the reagents.
2. Adding the necessary reagents without danger of contamination by oxygen.
3. Taking samples at intervals during the experiment.
4. Continuous observation of changes produced in the reaction of the solution by such processes as respiration and photosynthesis.

THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

V. THE ADDITIVE EFFECT OF SALT AND BASE AND THE ANTAGONISTIC EFFECT OF SALT AND ACID.

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(Received for publication, September 20, 1917.)

I. Introductory Remarks.

In former papers[1] it has been shown that the washed membranes of the egg of *Fundulus* are impermeable for potassium salts, that the addition of a moderate amount of a second salt accelerates the diffusion, and that an addition of a greater quantity of a second salt inhibits the diffusion of potassium salts into the egg. When alone in solution potassium salt cannot diffuse through the membrane of washed eggs until the potassium salt itself has supplied the "salt effect." It was pointed out that these effects of salt on diffusion were analogous to the effects of salts on globulins, which are insoluble in pure water, soluble in a moderate concentration, and insoluble again in a very high concentration of salt.[2] This would make it appear as if the diffusion of potassium salts depended on the solution of a certain membrane constituent, with qualities resembling those of a globulin. Potassium was used in these experiments since it causes cessation of the heart beat of the embryo as soon as a certain quantity has diffused into the egg. The cessation of the heart beat was, therefore, a convenient indicator for the diffusion of a certain quantity of potassium into the egg.

It seemed desirable to test whether the analogy between the behavior of globulins and the diffusion of electrolytes through

[1] Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339, 353, 363; 1916-17, xxviii, 175.

[2] Loeb, *J. Biol. Chem.*, 1917, xxxi, 343.

the membrane could be carried further, and for this purpose the effect of neutral salts on the diffusion of acids and alkali was examined. Hardy states that there exists an antagonism between the solvent actions of salts and acids while the solvent actions of salt and base are additive. From this difference Hardy has drawn interesting inferences on the manner of combination between globulins, salts, acids, and alkali.

"There is however one feature of fundamental importance which is never obscured, and that is the antagonism between the solvent actions of salts and acids, and the additive nature of the combined solvent action of salts and alkali. This feature arises I believe from the fact that acid globulin is insoluble by salt. Salts will combine with globulin or with alkali globulin to form soluble compounds; they will not so combine with acid globulin.

If this be true, then, when acid globulin is precipitated and redissolved by salt, the acid must be displaced and the globulin redissolved, not as salt-acid globulin but as salt globulin. The displacement of the acid can readily be followed by methyl orange. When HCl globulin is precipitated by a salt this indicator shows that free acid is liberated. But I failed completely to detect the liberation of free alkali when salt is added to a solution of alkali globulin. This suggests that precipitation of acid globulin is partly a definite chemical replacement of acid by salt,

$$GHS + BS = GBS + HS.$$

The fact that alkalies slightly assist solution in salts while acids very generally depress it suggests an interesting possibility, namely, that in the compounds GHS and GBS the acid HS and the salt BS are united to the molecule of G in the same way, so that they compete with one another, while in GB and GHS the base and salt are united to different parts of the molecule."[3]

It was, therefore, expected that if the analogy between the action of electrolytes upon the solution of globulin on the one hand, and the diffusion through the membrane on the other, would hold, that the addition of a salt should increase the rate of diffusion of alkali into the egg and diminish the rate of the diffusion of acid into the egg. This should show itself in this way, that a neutral salt would increase the toxicity of a base and diminish the toxicity of an acid. This is actually the case, as will be shown in this paper. It was necessary to make sure that the salts used were neutral or at least not alkaline, since

[3] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 323–24.

if they were alkaline the additions of salt to acid would make the acid weaker and when added to alkali increase the alkalinity. This would indeed result in an apparent antagonism of salt to acid and an apparent additive effect of salt to base. Hence to prove a real antagonism between acid and salt it was necessary to make sure that the salt solutions were not alkaline. Tests were made with phenolphthalein, neutral red, and congo red. The neutral red tests showed that the salt solutions used (with the exception, of course, of sodium citrate) were all slightly on the acid side of neutrality (all being as red as or more than distilled water). A trace of alkali sufficed to bring them to neutrality. Thus, one drop (0.05 cc.) of N/100 NaOH sufficed to turn 50 cc. of M/4 NaCl or M/32 $CaCl_2$ or M/32 $SrCl_2$ from red to yellow (with neutral red as an indicator). Na_2SO_4 was more decidedly acid, 0.5 cc. N/100 NaOH being required to turn 50 cc. M/32 Na_2SO_4 yellow. We are, therefore, dealing in the following experiments with an effect of the neutral salt.

The addition of a neutral salt to an acid with different anion has no effect on the hydrogen ion concentration. Dr. Haas was kind enough to compare with the aid of his indicators the H ion concentration of M/500 acetic acid made up in H_2O and M/32 Na_2SO_4, and found it identical, namely 3.6×10^{-5}.

II. The Antagonistic Character of the Combined Action of Salts and Acid.

Loeb and Wasteneys[4] have published two papers showing that the adult fish of *Fundulus* can resist acid much better if some neutral salt (NaCl or $CaCl_2$) is added to the acid. Acid killed these fish by altering the surface of their gills whereby respiration became impossible. The effect of the acid on the external surface of the fish was directly visible inasmuch as the outer lining of the body became white and pealed off. The presence of an adequate amount of salt prevented this effect of the acid when the latter was not too concentrated.

2 years ago the writer published similar experiments on the effect of neutral salt on the rate of diffusion of acid into the egg

[4] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

of *Fundulus*.[5] The acid had to diffuse through the membrane in order to reach the embryo. As soon as the acid penetrates in sufficient quantity into the egg the embryo begins to coagulate and become white, the thinnest part, the tail, showing this effect earlier than the rest. The heart is protected by the pericardium and therefore the heart beats may still be going on at a time when the tail is already coagulated by the acid. As soon as the heart stops beating recovery is no longer possible, since by this time the whole body is coagulated and for this reason the cessation of the heart beat could in this case be used as the criterion for death.

It was found that neutral salts inhibit the rate of diffusion of acid through the membrane. This could be proved by the fact that rhodanates and tartrates prevented the toxic action of acid upon the embryo *inside* the egg, while these salts had no such action upon the fish when outside the egg, the salts themselves being too toxic to be of any use for the fish itself. They were, however, of use in acting upon the external surface of the membrane of the egg, thereby retarding the diffusion of acid through the membrane.

The inhibiting action of salts on the rate of diffusion of acid through the membrane is a function of the anions as well as of the cations. Organic sodium salts antagonize acid better than the inorganic ones and the bivalent anions better than the monovalent ones. Likewise Ca and Sr antagonize better than the univalent cations and also better than Mg and Ba.

The relative efficiency of cations in antagonizing acid is shown in the following table, giving the *minimal* concentration of different neutral salts which permits 50 per cent of the embryos to survive in M/500 acetic acid after 18 hours.

LiCl	Die in all concentrations.
NaCl	" " " "
RbCl	" " " "
CsCl	" " " "
$MgCl_2$	" " " "
$CaCl_2$	M/2048
$SrCl_2$	M/512
$BaCl_2$	M/32

[5] Loeb, *J. Biol. Chem.*, 1915, xxiii, 139.

The superior antagonistic effect of the bivalent over the univalent cations is beyond doubt, as is also the difference between Ca and Sr on the one hand and Mg and Ba on the other.

As far as the anions are concerned, the difference in the antagonistic action between Cl and SO_4 is marked and independent of the nature of the cation. Table I gives the percentage of surviving embryos in M/500 acetic acid in M/8 chlorides and sulfates of different cations.

TABLE I.

After.	Percentage of surviving embryos in M/500 acetic acid made up in chlorides and sulfates of different cations.		
	Cation.	Cl	SO_4
hrs.			
24	Li	0	80
24	Na	0	100
8	NH_4	0	80
24	Mg	0	100

The sulfates are so much more powerful in their antagonism to acetic acid than the chlorides that at the time when the embryos had all been killed in the chloride solution they were still all active and alive in the sulfate solution.

In order to get a quantitative expression of the relative efficiency of univalent and bivalent anions the embryos were put into M/500 acetic acid solution to which various concentrations of different sodium salts were added. That concentration of these salts was ascertained which allowed 50 per cent of the eggs to be alive after 10 hours. This concentration was found to be for

$NaCl$ M/4
$NaBr$ M/4
$NaNO_3$ ..... Slightly > M/4
Na_2SO_4 ..... M/32

When we add increasing quantities of salt to an acid very soon a concentration is reached where the further addition of salt accelerates the action of the acid; this action takes place at a concentration of the salt where the salt alone is not yet very harmful.

TABLE II.

	Percentage of surviving embryos after 6 hrs. in M/500 acetic acid made up in													
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	H_2O
NaCl	0	40	100	75	35	5	5	0	5	0	0	0	5	0
NaBr	50	90	95	50	10	0	0	15	5	0	10	5	0	0
$NaNO_3$	15	95	95	40	20	15	5	0	0	10	0	0	5	0
Na_2SO_4		90	100	100	100	100	80	80	10	5	5	10	10	10

TABLE III.

	Percentage of surviving embryos after 10 hrs. in M/500 acetic acid made up in												
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H_2O
NaCl	0	10	60	30	0	0	0	0	0	0	0	0	0
NaBr	15	85	70	0	0	0	0	0	0	0	0	0	0
$NaNO_3$	0	80	40	5	0	0	0	0	0	0	0	0	0
Na_2SO_4		50	100	100	100	90	10	0	0	0	0	0	0

Especially after 10 hours it is clear that the antagonistic effect of M/1 solutions of NaCl, NaBr, and $NaNO_3$ is less than that of M/2 or M/4 solutions. When the salt concentration exceeds a certain limit its antagonistic action to acid diminishes again.

In a previous paper it was pointed out that when eggs are treated with a low concentration of salt and are *afterwards* exposed to acid the diffusion of acid may be accelerated. Such an effect was never observed when acid and salt were simultaneously in solution no matter how low the concentration of the salt.

III. The Additive Character of the Combined Action of Alkali and Salt.

In working with alkali we have to remember that the CO_2 of the air as well as that produced by the living organism steadily diminishes the concentration of the alkali. This makes it necessary to carry on these experiments in small closed Erlenmeyer flasks in which the volume of solution is large compared

with the air space left and the volume of the eggs (20 in each flask). In addition it is necessary to restrict the experiment to short periods. At given intervals the eggs were taken out and examined and the percentage of embryos whose heart was still beating was determined. It was found that the salt always increased the efficiency of the alkali and in no case antagonized it. The following experiments with M/200 NaOH and M/200 tetraethylammoniumhydroxide may serve as an example. The solutions were made up in mixtures of NaCl, KCl, and $CaCl_2$ in the proportion in which these three salts exist in the sea water; namely, 100 molecules of NaCl to 2.2 molecules of KCl to 1.75 molecules of $CaCl_2$ (Table IV).

TABLE IV.

Solution.	Percentage of eggs surviving after 5 hrs. in various alkali solutions made up in NaCl + KCl + $CaCl_2$.										
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	H_2O
M/200 NaOH	0	0	0	10	30	45	80	100	100	100	100
M/200 tetraethylammoniumhydroxide	0	0	0	0	0	10	10	45	90	100	100

Even as low a salt concentration as M/32 has an accelerating effect upon the toxic action of alkali. It may be well to point out that these eggs can live for days in neutral solutions of NaCl + KCl + $CaCl_2$ as concentrated as 2.5 M.

In order to ascertain whether at least a trace of an antagonistic effect of salts upon alkali can be discovered, higher concentrations of the latter were used. The writer did not observe any indication of an antagonistic effect between a strong base and salt while the additive effect was always marked.

The next task consisted in ascertaining the influence of valency and nature of anions and cations of the salt on the additive effect. It was found that the same ions which had the greater antagonistic effects upon acid had the greater additive effect upon bases; *i.e.*, it required less of sulfate than of a chloride to accelerate the toxic effect of a base and less Ca than Na. In order to test this M/400 solutions of ethylamine were used. In a pure M/400

ethylamine solution the embryos live at least for a number of days. If we add, however, a neutral salt the same concentration becomes extremely toxic. If different sodium salts are added the toxicity of the solution increases with the valency of the anion, approximately in harmony with Hardy's exponential law. Table V gives the percentage of surviving embryos in M/400 ethylamine when made up in solutions of NaCl, Na_2SO_4, Na_2 tartarte, Na_3 citrate, $CaCl_2$, and $SrCl_2$ after about 22 hours.

TABLE V.

Solution.	Percentage of surviving embryos after 22 hrs. in M/400 ethylamine made up in various concentrations of salt solutions.										
	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
NaCl	30	50	75	70	75	70	95	90			95
Na_2SO_4	25	30	20	65	80	80	95	95			95
Na_2 tartrate	40	20	25	50	65	80	75				95
Na_3 citrate	0	0	0	10	5	30	70	80			90
$CaCl_2$	5	5	0	5	20	30	50	60	85	100	95
$SrCl_2$	25	30	30	35	55	100	85	95	85	90	95

Comparing the concentration of these salts which permits 50 per cent of the embryos to remain alive we find this to be for

NaCl	M/32
Na_2SO_4	M/128
Na_2 tartrate	M/128
Na_3 citrate	M/1024

The ratio of efficiency of monovalent to bivalent to trivalent anion is, therefore, as 1:4:32, while Hardy's rule demands in this case 1:4:16. The exception in citrate is probably due to the fact that citrate is in itself alkaline. In experiments of shorter duration the ratio between tartrate, sulfate, and citrate was M/128: M/128: M/512, which shows citrate to be four times as efficient as sulfate. In the writer's previous papers it was pointed out that Hardy's valency rule holds also for the influence of salts on the rate of diffusion of KCl into the egg.

As far as the cations are concerned, it is obvious that the addition of a slight amount of $CaCl_2$ accelerates the action of the

base considerably more than NaCl, M/1024 $CaCl_2$ being as efficient as M/32 NaCl. $SrCl_2$ is slightly less efficient than $CaCl_2$.

It is of interest that the addition of salts to NH_4OH gave no clear results; there was neither a clear additive effect nor an antagonistic effect between salt and NH_4OH.

IV. Proof That the Salt Action Described in This Paper Is Due to an Influence upon the Rate of Diffusion by Alkali and Acid Through the Membrane.

As stated in previous papers the experiments on the *Fundulus* egg have the striking advantage of demonstrating directly whether a salt action is due to an influence upon the rate of diffusion through a membrane or whether it is due to an action on the living protoplasm. The embryo lives inside the membrane of the egg from which it is separated by a watery liquid. By comparing the effect of a solute on the embryo while *in* the egg, and on the embryo *immediately after hatching*, we can make sure whether or not the effect observed on the egg is merely due to an influence upon the rate of diffusion through the membrane or to an effect upon the embryo inside the egg. When the difference in the two cases is merely one of degree a doubt might still be entertained; but when it becomes one of kind, as is the case in these experiments, all doubt vanishes.

The *Fundulus* embryo inside the egg remains alive indefinitely when the eggs are put into M/400 ethylamine but they all die in less than 24 hours when the M/400 ethylamine is made up in M/64 $CaCl_2$ or in some other salt. The salt accelerates death. When we make the same experiment on the newly hatched embryo we find that the latter dies in about 20 minutes in M/400 ethylamine and the addition of $CaCl_2$ does not accelerate the action but retards it markedly, the embryo living from 40 to 60 minutes in M/400 ethylamine made up in M/32 $CaCl_2$. The same is true for other salts, *e.g.*, Na_2SO_4 or $SrCl_2$; instead of accelerating the action of alkali on the hatched embryo they retard it. This effect of the salt then is exactly the reverse from the effect of the salt upon the embryo while *inside* the egg. This does not permit of any other interpretation than that the combined effect of salt and alkali upon the egg is due to an action

on the membrane surrounding the egg and not upon the embryo inside the egg. We must therefore conclude that the addition of a neutral salt to the alkali allows the latter to diffuse more rapidly into the egg. The embryo which is killed in 20 minutes in M/400 ethylamine, when brought directly into this solution, remains alive in this solution, as long as it is surrounded by the egg membrane, and the reason can only be that this solution never reaches the embryo as long as it is surrounded by the membrane. If, however, a salt of the proper concentration is added to the M/400 ethylamine solution the latter can diffuse into the egg. In a stronger ethylamine solution, *e.g.*, M/200, the embryos are all killed inside of 24 hours when inside the egg. In this case the higher concentration of the alkali itself supplies the "salt effect" upon the diffusion of base into the egg.

As far as the direct action of alkali on the embryo is concerned it is visibly due to the solution of the surface elements of the embryo. In a M/400 ethylamine solution the surface of the head is dissolved very quickly, leaving the eyes protruding.

We have already mentioned the fact that tartrates and rhodanates can be used to antagonize the action of acid on the egg, while they cannot be used to counteract the injurious effect of acids on the fish when outside the egg.[5] This shows that the antagonistic action of salt to acid, as long as the embryo is inside the egg, is due to an effect on the membrane of the egg and not on the embryo itself.

V. *Theoretical Remarks.*

We have pointed out in this and in a previous paper the analogy which exists between the conditions for the diffusion of electrolytes through the membrane of the *Fundulus* egg and the condition for the solution of globulins. It seems on the whole that those conditions which tend to make globulins soluble also permit or accelerate the diffusion of certain electrolytes through the membrane of the egg of *Fundulus;* while those which render globulins less soluble also inhibit or retard the diffusion of certain electrolytes. Thus it was shown in a former paper that potassium salts diffuse through the membrane of *Fundulus* when the membrane has had a chance to combine with

a certain moderate quantity of salt; while either a membrane free from salt or one having an excess of salt is more or less impermeable for the same potassium salt. This corresponds to the fact that globulins are soluble in a moderate amount of salt but insoluble in distilled water or in an excess of salt. It was also pointed out that the valency rule found by Hardy for the precipitation of globulins holds also for the action of salt on the diffusion of potassium through the membrane of the *Fundulus* egg. It was pointed out, however, that there exists merely an analogy and not an identity in the diffusion of salts through the membrane and the solubility of globulins; since the ratio for the solvent and precipitating effect of a neutral salt on globulins differs widely from the ratio for the accelerating and inhibiting effect of the same salt for the diffusion through the membrane.

In this paper the analogy is carried further. Salts and acids have an antagonistic and salt and bases an additive effect upon the solution of globulins. It could be shown that the relation holds for the diffusion of acid and bases through the membrane. Salts accelerate the diffusion of bases and retard the diffusion of acid through the membrane. The analogy apparently breaks down again through the fact that the increasing valency of both cations and anions of salts increases the antagonistic action to the diffusion of acid and increases the accelerating action on the diffusion of alkali.

Salts, therefore, increase the rate of diffusion of certain electrolytes through the membrane of the egg of *Fundulus* when they are also able to dissolve globulins; and they retard or prevent the diffusion when they are likely to prevent the solution of globulins. The facts, however, do not permit us to say that the substance in the membrane upon which the diffusion of electrolytes depends *is* a globulin. A difference in the action of salts on acids and bases was also observed by the writer in his old experiments on the swelling of muscle, where he found an antagonistic action of neutral salts to acid but not to base.[6]

Some authors assume that the proof of an increase in permeability of a membrane to *one* substance indicates an increase of permeability to *all* substances. The fact that salts increase

[6] Loeb, *Arch. ges. Physiol.*, 1899, lxxv, 303.

the permeability of the *Fundulus* membrane to alkali while they have the opposite effect on the permeability to acids should warn us against such generalizations.

SUMMARY OF RESULTS.

1. When *Fundulus* eggs are put into solutions of bases not sufficiently concentrated to injure the embryo the bases become injurious when neutral salts in low concentration are added. This additive effect between salts and bases was observed also at higher concentrations of the base.

2. When *Fundulus* eggs are put into an acid which kills the embryo rapidly the addition of a neutral salt has an antagonistic effect. The same antagonism between acid and salt was observed also for lower concentrations of acids.

3. The minimal quantity of a salt required for these effects diminishes with an increase in the valency of both anion and cation of the salt. In the case of the anion the quantity diminishes according to Hardy's valency rule. In addition the chemical nature of the salt is of importance.

4. It can be shown that the additive effects of salt to base and the antagonistic effect of salt to acid are due in the case of the egg of *Fundulus* to an influence of the salt upon the rate of diffusion of alkali and acid through the membrane of the egg; this influence being accelerating for the diffusion of alkali and retarding for the diffusion of acid.

5. The partial similarity of the action of neutral salts in these cases with the action of salt on the solution of globulins in the presence of acid and alkali is pointed out.

THE DETERMINATION OF SMALL AMOUNTS OF CALCIUM, PARTICULARLY IN BLOOD.

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The determination of calcium in small amounts of blood and other biological materials low in this element involves the solution of three main problems. The first is the preparation of a solution containing all of the calcium but free from interfering organic matter. The second is the precipitation of the calcium in the presence of such other elements as commonly occur, while the third consists in the accurate estimation of this precipitated calcium.

Toward a solution of these problems the present authors have suggested the precipitation of calcium as oxalate directly in the filtrate from biological fluids after removal of protein, the final estimation being carried out by a refinement of the permanganate titration method (1). In this paper is presented in greater detail the procedure which our experience with this method has suggested as well as data with regard to the accuracy of estimation. It is our belief that in many cases the substitution of deproteinization for ashing marks a distinct advance, and that evidence as to the accuracy with which minute amounts of calcium may be estimated by permanganate titration will show this titration to have a wider applicability than that with which it has generally been credited.

Methods.

I. Procedure for Serum, Plasma, and Whole Blood.

A. Removal of Protein.—Whole blood is preserved with powdered sodium citrate to make approximately 1.5 per cent. An additional 1 per cent of citrate should be added to plasma if this

is not to be analyzed at once. Directions given below are for serum or plasma. Twice the quantity of whole blood should be employed and reagents increased proportionately.

Pipette 5 cc. of serum[1] or plasma into a 50 cc. volumetric flask containing exactly 20 cc. of distilled water. Rinse by once drawing the solution up into the pipette. While rotating the flask add from a pipette 5 cc. (1 cc. per cc. of plasma or serum) of a 4 per cent solution of sodium picrate. In the same manner add slowly 5 cc. of hydrochloric acid (1:2). Heat in a boiling water bath with occasional rotation for 15 minutes. Cool to a little below room temperature in cold water. Pour onto a folded calcium-free filter paper and allow to drain well.

B. Precipitation of Calcium.—Measure an aliquot (usually 25 cc.) of the filtrate into a 50 cc. Erlenmeyer flask (Pyrex). Neutralize cautiously with concentrated ammonium hydrate added drop by drop from a burette, using one or two drops of alizarin indicator solution (0.2 per cent). Titrate back until faintly acid with approximately 0.5 N hydrochloric acid. McCrudden's method is then followed. Add from a burette 2.5 cc. of the hydrochloric acid 0.5 N mentioned above and then the same amount of 2.5 per cent oxalic acid. To the boiling solution add dropwise in two portions 2.5 cc. of 3 per cent ammonium oxalate. Digest at near boiling for 15 minutes.

Cool in ice water to room temperature or lower. Add another drop of alizarin and also dropwise from a burette 2.5 cc. of 20 per cent sodium acetate solution while rotating the flask, or if found necessary add until the alizarin just *begins* to change color. Allow to stand over night (or at least 4 hours after 10 minutes' shaking).

Transfer completely to a 50 cc. round bottom centrifuge tube with the aid of a little water and whirl for about 3 minutes at 1,500 revolutions per minute. With an automatic siphon (Fig. 1) draw off the supernatant liquid at first rapidly and then gently to within a drop or two. Wash with cold distilled water

[1] If more than 5 cc. of serum is available the amount may be increased as the accuracy of the method increases somewhat with the amount of serum used. For instance 7 cc. of serum may be diluted to 50 cc. and 40 cc. of filtrate employed. For amounts smaller than 5 cc. the ashing procedure may be used.

(15–20°C.) first adding about 20 cc., washing down the sides and rotating the tube. Then add more water from the wash bottle to within about 1.75 cm. of the top of the tube (approximately 50 cc.). Placing the metacarpal portion of the palm of the hand at the thumb over the mouth of the tube shake vigorously for 5 to 10 seconds. Centrifuge again. Siphon off to within one or two drops and titrate.

C. Titration.—To the precipitates in 50 cc. centrifuge tubes add, with shaking, 4 cc. of 5 per cent sulfuric acid (very faintly

FIG. 1.

tinged with potassium permanganate). Place in a water bath at 65°C. until the tubes approach the temperature of the bath. Remove and titrate rapidly with an approximately 0.0133 N potassium permanganate solution, with moderate shaking using a white background. A burette of 10 cc. capacity with which readings can be readily estimated to 0.01 cc. is desirable.

The end-point is attained when a faint but definite pink color persists for a minute or longer on gentle shaking and standing. If the precipitate has not been contaminated the end-point will

be sharp to 0.01 cc. The sulfuric acid must be brought in contact with all parts of the tube as far up as the original solution extended. The burette reading should be corrected for the small amount of permanganate required to titrate 4 cc. of the sulfuric acid to the same end-point.

D. Calculation.—1 cc. of 0.0133 N potassium permanganate is equivalent to 0.267 mg. of calcium. The exact factor for a given solution must be determined by standardization. Multiply the number of cc. used by this factor to obtain the amount of calcium in the 25 cc. of filtrate. If blank on reagents is not negligible deduct. Multiply by 28 to get mg. of Ca per 100 cc. of serum or plasma.

II. Procedure for Human and Cow's Milk.

For human milk dilute 5 cc. with an equal volume of water and treat 5 cc. of the mixture in exactly the same way as serum (Procedure I, above). The deproteinization procedure is here advantageous because of the relatively low calcium content of this fluid (about 30 mg. per 100 cc.).

For cow's milk dilute 5 to 50 cc. and treat 5 cc. of the mixture in the usual manner (Procedure I). Here deproteinization has less advantage over ashing.

III. Procedure for Cerebrospinal Fluid.

10 cc. of spinal fluid in a 25 cc. volumetric flask are treated with 10 cc. of water and 1 cc. of sodium picrate solution and then 1 cc. of concentrated hydrochloric acid is added slowly with rotation. The flask is then made up to mark, heated in a boiling water bath for 15 minutes, cooled, filtered, and an aliquot, usually 20 cc., used.

IV. Procedure for Urine.

Where small amounts of urine only are available or where the highest percentage accuracy is not required the following procedure has been found advantageous.

10 cc. of filtered urine (in exceptional cases 20 cc.) are introduced into a 50 cc. Erlenmeyer flask. Concentrated urines should be diluted with an equal volume of water. The calcium

is then precipitated following exactly McCrudden's procedure (2) merely using correspondingly smaller amounts of reagents. The sodium acetate solution should be added with great care. Centrifuge and wash in the manner indicated for blood serum.

In the precipitation, by shaking 10 minutes and allowing to stand for 4 hours, there is little danger of precipitating uric acid which must be absent in the titration. If more convenient however, allow to stand over night. If uric acid separates the oxalate may usually be washed out from the flask with the aid of small portions of water, leaving the much larger and heavier uric acid crystals behind.

V. Determination in the Ash from Biological Materials.

The ash is dissolved by digesting with dilute hydrochloric acid for about 30 minutes. Neutralize with ammonia and precipitate the calcium in the usual way using amounts of reagents proportional to the volumes of the solutions.

Reagents.

Potassium Permanganate 0.0133 N.—Dissolve 0.50 gm. of pure potassium permanganate crystals in 1 liter of redistilled water in a thoroughly clean Florence flask which has been rinsed with the same water. Digest at near the boiling point for 36 hours. A funnel covered with a watch-glass may be used as a reflux condenser. Cool and allow to stand over night. Without disturbing the sediment of manganese oxides filter with gentle suction through a 3 inch Buchner funnel lined with ignited asbestos. Both funnel and flask should be rinsed with redistilled water. Transfer the permanganate solution to a glass-stoppered bottle free from traces of organic matter and keep in the dark when not in use.

This permanganate solution may be conveniently standardized against oxalic acid (0.1101 gm. pure crystals to 100 cc.) or sodium oxalate of similar strength. To 10 cc. of the oxalic acid solution (equivalent to 3.5 mg. of Ca) in a 50 cc. Erlenmeyer flask add 10 cc. of 10 per cent sulfuric acid which has been treated with just sufficient permanganate solution to give it a faint pink color. Place in a water bath at 65°C. for a few minutes. Remove and titrate at once to a definite pink color which persists for at least a minute. If kept in a dark place the oxalic acid solution does not lose appreciably in strength in from 10 days to 2 weeks. Ordinarily the permanganate solutions after they have stood for several days will not vary over 0. 1 per cent a week. On account of the sensitivity of the reagent and as a control on technique it is, however, desirable to run a check with each series of determinations.

Sodium Picrate Solution, 4 per Cent.—To 40 gm. of dry purified picric acid[2] add a little calcium-free water and 10 gm. of highest purity anhydrous sodium carbonate (calcium-free) dissolved in 50 cc. of water. Dilute to 1 liter. Shake until the picric acid is completely dissolved. Add concentrated hydrochloric acid until a slight permanent precipitate of picric acid forms. Filter through highest grade filter paper.

Sodium Alizarin Sulfonate Solution, 0.2 per Cent.—An aqueous solution. There is no especial difficulty in using this indicator in the presence of picric acid.

Hydrochloric Acid, Approximately Half Normal.—Dilute 40.5 cc. of 36 per cent hydrochloric acid to a liter.

Oxalic Acid Solution, 2.5 per Cent.—Let stand over night and filter if necessary.

Ammonium Oxalate Solution, 3 per Cent.—Let stand over night. Filter off the precipitate.

Sodium Acetate Solution, 20 per Cent.—The crystallized salt is used. Filter and add a few cc. of chloroform as a preservative.

Sodium or Potassium Citrate, Crystallized.—This as well as the other chemicals used must be free from all but the most minute traces of calcium. Ordinary C.P. grades must be recrystallized.

Apparatus.

In addition to the centrifuge and tubes the only special apparatus required is a simple automatic siphon of the type illustrated in Fig. 1. We have found an apparatus of this type necessary to facilitate the rapid and complete removal of supernatant liquid and wash water after centrifugation. Unless very gentle suction is employed there is great danger of loss due to disturbance of the finely crystalline precipitate. It is also necessary to draw off all but one or two drops of fluid in order to avoid an additional washing to remove the last traces of soluble oxalate.

An electric bulb is placed just above the arm A (Fig. 1) and a black cardboard extending from slightly above A to below D is attached to the right arm of A so as to leave the left arm unhampered. 4 mm. glass tubing is used. The tip of D is slightly curved as shown in the cut, aiding greatly in removing the last few drops of liquid without disturbing the precipitate. A

[2] Picric acid may be purified as follows. To 50 gm. of picric acid add 700 cc. of distilled water. Boil until clear, and while boiling add 10 cc. of concentrated hydrochloric acid. Cool. Wash by decantation with 100 cc. of distilled water. Repeat the recrystallization. Transfer to a Buchner funnel and wash with about 150 cc. of water. Dry in a desiccator.

rubber connection is used for D in order to give slight flexibility and facilitate exchange of tips. The siphon is filled by opening the upper stop-cock, and in siphoning the lower stop-cock is used to regulate the flow.

If any trace of precipitate is accidentally drawn into D or A it at once becomes visible and by closing the lower and opening the upper pinch-cock can be washed back into the tube. In a similar way the siphon may be rinsed and centrifugates separated quantitatively for further examination. The reservoir is kept filled with redistilled water preserved with chloroform. A glass stop-cock may be conveniently substituted for the lower pinch-cock.

DISCUSSION.

Citrate is used to prevent clotting because it is satisfactory and more available than hirudin. The presence of the amounts used we have found by many determinations not to interfere in any way with the precipitation of calcium. Neither is any calcium precipitated out from plasma by citrate as it is with oxalate. Diluting citrated whole blood with water to three volumes and adding a few cc. of chloroform aids in its preservation for longer periods of time. Dilution of plasma with several volumes of water after the further addition of citrate was also found useful but it did not keep as long as whole blood.

The usual gravimetric determination of calcium when applied to the ash of small amounts (25 cc. or less) of blood is inaccurate for several reasons. Aside from the limitations of weighing which under these conditions will ordinarily involve a high percentage error, it is also necessary to carefully reprecipitate the oxalate or very high results may be obtained due to occlusion, silica here causing most of the difficulty.

Another source of error lies in ashing the blood or serum, when this is carried out in the usual way. The greater the amount of blood or serum taken for ashing the more serious does this error become. The ashing of 25 cc. of blood is not only tedious but the losses of calcium are usually quite marked. By decreasing the amount of serum to 10 cc. or less, ashing is facilitated and losses are reduced. Ignition is also facilitated by evaporation in a Freas oven over night at 90–95°C. which gives a porous

protein mass readily and completely burned off by the aid of a 3 inch microburner applied carefully at one side. The ignition may also be completed in an electric oven. The hot plate should not be used as it bakes the protein into a hard mass and causes an incrustment of the salts which makes ignition difficult. Good results may be obtained by ashing small amounts of serum as indicated, but care is required and the tendency to run low must be borne in mind.

In our earlier work a preliminary washing with 0.12 per cent ammonium oxalate solution was used but this proved unnecessary as a single washing with water is sufficient to remove soluble oxalate if the centrifugates are drawn off completely as directed. This latter is, however, quite necessary as otherwise an additional washing would be required. The solubility of calcium oxalate in the wash water is negligible if centrifugation is carried out promptly. The flasks in which the precipitation is carried out and the centrifuge tubes should be scrupulously clean and bright and no dust or organic matter must be permitted to enter them.

The preparation of weak standard permanganate by dilution of stronger solutions even if the reagent is used at once leads to inaccurate results as we have pointed out elsewhere (3). A more concentrated sulfuric acid or a much higher temperature in the titration must also be avoided as these lead to high results and an indefinite end-point. Under the conditions of the method the end-point offers none of the difficulties usually described. If a diverging result is obtained in a series of determinations the possibility of organic contamination may be checked up by ashing the titration fluid and reestimating the calcium. In the absence of contamination identical results will be obtained. In this way we have also shown that no contamination of the oxalate occurs if ordinary precautions are observed (Table II).

The advantage of deproteinization over ashing methods in the analysis of blood for inorganic elements lies in the great saving of time where numerous determinations must be carried out. Prior to its use by us for calcium and magnesium this principle had been used by Greenwald (4) in the determination of acid-soluble phosphorus and had been shown by McLean and Van Slyke in the case of chlorides (5) to be applicable where a high degree of accuracy is desired.

Picric acid is a satisfactory protein precipitant, is readily purified, can be used in hot solution containing mineral acid, and being less soluble in the presence of acid is in great part removed before the precipitation of the calcium. Our data show that calcium is readily and completely precipitated in the presence of the amounts of picric acid found in our filtrates. High concentrations slow the reaction, so that in running a blank on this reagent it should be ashed.

The precipitation of the calcium is based on McCrudden's procedure. The ammonium oxalate is added to keep the concentration of oxalate ions high. It is safer to let stand over night for precipitation.

In Table I are given the results of a series of determinations by the permanganate titration method upon pure solutions containing besides calcium and magnesium relatively large amounts of phosphates and iron. In the Solution I the amounts of these interfering constituents are far greater than those tested by McCrudden as given in his original papers, and inasmuch as tissues anywhere near as high as this in iron are much lower in phosphorus the conditions of precipitation are much more difficult than those we would expect to meet with in the analysis of the ash of any ordinary biological material.

The results show that calcium may be accurately determined under these extreme conditions. They cannot be taken as representing the attainable accuracy, but inasmuch as no particular effort was made to adapt the determination to the widely varying amounts of calcium present, the results illustrate the flexibility naturally associated with a titration method.

In Table II are given the results of a series of determinations made upon serum, plasma, whole blood, cerebrospinal fluid, human milk, and human semen. In some cases the protein precipitate was ashed and calcium determined as a second check upon the uniformity of the calcium distribution. This point was also checked by determinations on serum to which known amounts of calcium were added. Direct checks on the purity of the calcium oxalate precipitates obtained from the various biological fluids are given in Nos. 36 to 43. These are of course also checks upon the whole precipitation and titration procedures. That the results obtained by deproteinization are in

general slightly higher than those by ashing, can, in view of the direct checks on calcium distribution, only be due to the tendency of the latter to run a trifle low. While figures are given for calcium in 100 cc. of serum, etc., it must be borne in mind that the actual determinations represent from 0.3 to 1.0 mg. of calcium.

The four determinations upon human semen besides illustrating the applicability of the method are of interest as revealing the relatively high calcium content of the fluid in question, this varying apparently with the content of the spermatozoa high in nuclein.

With ordinary care and using the amounts indicated in the methods results within 2 per cent of the true values may be regularly obtained. The method possesses no fixed limitations as to

TABLE I.

Estimation of Small Amounts of Calcium in the Presence of Relatively Large Amounts of Magnesium, Iron, and Phosphates.

No.		Solution taken.	Ca	
			Found.	Theory.
		cc.	*mg.*	*mg.*
1	Solution I (in 100 cc.: Ca 7.28 mg., MgO 10 mg., Fe_2O_3 50 mg., P_2O_5 100 mg.).	100	7.30	7.28
2	" "	100	7.29	7.28
3	" "	100	7.25	7.28
4	" "	25	1.83	1.82
5	" "	25	1.81	1.82
6	" "	25	1.83	1.82
7	" "	10	0.728	0.728
8	" "	10	0.733	0.728
9	Solution II (in 100 cc.: Ca 7.17 mg., MgO 5.0 mg., Fe_2O_3 2.0 mg., P_2O_5 20 mg.).	100	7.16	7.17
10	" "	100	7.18	7.17
11	" "	25	1.79	1.79
12	" "	25	1.82	1.79
13	" "	7	0.507	0.502
14	" "	7	0.507	0.502
15	" "	4	0.290	0.287
16	" "	4	0.292	0.287
17	" "	2	0.143	0.143
18	" "	2	0.145	0.143

TABLE II.

The Determination of Calcium in Blood Serum and Other Biological Fluids.

No.		Ca per 100 cc.	
		By ashing.	By deproteinization.
		mg.	*mg.*
1	Ox serum No. 8.	10.16	10.22
2	" " " 8.	10.08	10.01
3	" " " 10.	10.47	10.48
4	" " " 10.	10.50	10.57
5	" " " 10.		10.52
6	" " " 10.		10.30
7	" " " 10. Calcium determined in both filtrate and precipitate.		10.46
8	" "		10.47
9	Ox serum No. 10. 0.05 mg. Ca added per cc.		15.37
10	" " " 10. 0.05 " " " " " "		15.23
11	Human serum (eclampsia).	8.47	8.52
12	" " "		8.66
13	Ox plasma.	8.80	8.87
14	" "	8.80	8.95
15	" blood, whole, No. 10.	6.40	6.53
16	" " " " 10.	6.30	6.56
17	" " " " 10. Calcium determined in both filtrate and precipitate.		6.54
18	" "		6.62
19	Cerebrospinal fluid, human.	5.13	5.03
20	" " "	5.01	5.00
21	" " "	5.04	4.99
22	Human milk No. 9.	27.63	28.00
23	" " " 9.	28.16	28.28
24	" " " 10.	32.02	32.89
25	" " " 10.	32.70	33.00
26	" " " 11.	20.65	
27	" " " 11.	20.67	
28	" " " 12.	32.61	
29	" " " 12.	32.19	
30	" " " 13.	35.10	
31	" " " 13.	35.30	
32	" semen No. 1, Subject A. Ca 3.02 per cent of ash.	20.60*	

*Mg. per 100 gm.

TABLE II—*Concluded.*

No.		Ca per 100 cc.	
		By ashing.	By deproteinization.
		mg.	*mg.*
33	Human semen No. 2, Subject A. Ca 2.36 per cent of ash.	20.60	
34	" " " 3. " " 2.60 " " "	23.40	
35	Human semen No. 4, Subject B.	84.50	
36	Whole blood No. 8. Titration fluid ashed and calcium reestimated.	6.40	6.40
37	" " 7. "	6.01	6.05
38	" " 9. "	9.80	9.87
39	Serum " 11. "	10.00	10.10
40	Plasma " 10. "	8.15	8.30
41	Human milk " 2. "	28.28	28.45
42	Cerebrospinal fluid No. 3. "	6.05	6.10
43	" " " 4. "	5.25	5.34

accuracy, but this depends on well known factors, primarily upon the care exercised and the amounts of calcium determined. Hence the principles and technique of these methods are adaptable to widely varying needs.

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BIBLIOGRAPHY.

1. Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1916, xxiv, p. xxii; 1917, xxix, 337. Halverson, J. O., Mohler, H. K., and Bergeim O., *J. Am. Med. Assn.*, 1917, lxviii, 1309.
2. McCrudden, F. H., *J. Biol. Chem.*, 1911–12, x, 187.
3. Halverson, J. O., and Bergeim, O., *J. Ind. and Eng. Chem.*, (in press).
4. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.
5. McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

THE CALCIUM CONTENT OF THE BLOOD SERUM IN CERTAIN PATHOLOGICAL CONDITIONS.

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In the tables which follow data are presented with regard to the calcium content of human blood serum in certain pathological conditions, particularly nephritis, as well as of the blood serum of normal men. The blood corpuscles are very low in calcium as has been shown by Abderhalden and others, so that any significant changes in blood calcium values will be shown by plasma values. It might appear that determinations on plasma would be preferable to those on serum but we have found changes in plasma values to be closely reflected in serum values even in uremia, where the calcium content of the blood is most markedly altered. Serum has therefore been employed as a matter of convenience.

Lyman (1) has recently presented data with regard to the calcium content of the whole blood in a considerable number of pathological conditions. This author is unable to account for the marked variations in blood calcium values obtained by him, for while the statement is made that the calcium varies but little in the great majority of subjects, values ranging from 5.2 to 9.6 mg. per 100 cc. are given for apparently normal individuals. Average figures of 6.1 for males and 7.1 for females are obtained, which the author considers a sufficiently great difference to stimulate further investigation. In another case with hemoglobin 160 per cent of standard a value of 2.8 mg. of calcium was found in the blood.

That these differences do not necessarily indicate any significant alteration in calcium metabolism but may be largely accounted for by variations in the ratio of plasma to corpuscles is apparent when we bear in mind the low calcium content of the corpuscles and the extreme variations which the ratio may undergo. The volume of plasma per unit of whole blood has also been shown to be on the average about 10 to 20 per cent greater for women than for men, though of course not constant for the

sexes. The very high and low values obtained by Lyman are thus most readily accounted for as representing respectively anhydremia and anemia or chlorosis. This view is confirmed by the low finding in dysentery with which is associated withdrawal of water from the blood, as well as by high values in cases where hemorrhage might be expected. That considerable percentage variations in blood count or hematocrit readings may occur among individuals considered normal, and that these are influenced by such physiological conditions as physical exercise, perspiration, fright, partial asphyxiation, age, the ingestion of food, changes in climate or altitude, etc., is well known.

In uremia decreases in plasma calcium and hematocrit readings frequently occur. The normal values obtained by Lyman in nephritis and other conditions might thus represent a compensation of these two factors. In view of these difficulties which are not met with in the case of plasma or serum, the determination of calcium in whole blood in disease loses most of its significance unless hematocrit determinations are also carried out, in which case the error of the latter method (an approximate one) is thrown upon the calcium determination.

The present authors have already presented data with regard to the calcium content of the blood in various stages of pulmonary tuberculosis (2). The methods used were those previously described by two of us (3). In Table I figures are given for the calcium content of human serum in a variety of conditions. For normal men values from 9.6 to 10.8 mg. with an average of 10.2 were obtained. That these values are fairly characteristic is supported by the finding of similar values in the great majority of pathological cases not known to be associated with disturbances in calcium metabolism. Thus a similar range was found for tuberculosis and may be noted for most of the cases here tabulated. This is true even of Cases 22 and 23 (Table I) where the clotting power of the blood was markedly disturbed. On the other hand an undoubtedly low value was obtained in one of two cases of eclampsia. A decrease in calcium was also noted in hematogenous jaundice. Less marked decreases were found in other forms of jaundice studied and in a case of pneumonia.

In Table II are presented data from a number of cases of nephritis of different types and degrees of severity, as well as cases of edema without nephritis, and of pernicious vomiting of pregnancy. In most cases urinary analyses were carried out in the attempt to establish some relationship between the acid, ammonia, and particularly calcium excretion in the urine and the

level of plasma calcium. Total acidity, hydrogen ion concentration, and ammonia were determined by usual methods and the urinary calcium by the micro method previously described (3), albumin being first removed.

TABLE I.

Calcium Content of Human Blood Serum in Certain Pathological Conditions.

No.	Sex.		Ca (mg. per 100 cc. of serum).
1	M.	Normal.	9.6
2	"	"	9.9
3	"	"	9.9
4	"	"	10.1
5	"	"	10.3
6	"	"	10.3
7	"	"	10.5
8	"	"	10.8
9	"	Syphilis, Wassermann positive + 4.	9.4
10	"	" " " + 4.	9.7
11	"	" " " + 3.	10.8
12	"	" " " + 2.	10.3
13	"	Jaundice, hematogenous; serum cholesterol low.	7.8
14	F.	" severe; serum cholesterol high.	9.2
15	"	" catarrhal; cholesterol normal.	9.2
16	"	" marked; with pancreatitis.	9.4
17	"	Eclampsia.	8.5
18	"	" 15 days after parturition; daily urinary Ca excretion, 0.03 gm.; ammonia 0.5 gm.	9.6
19	M.	Osteoarthritis, chronic. Ca whole blood, 6.8.	10.4
20	"	Osteomyelitis, chronic.	9.7
21	"	Hodgkins' disease.	10.8
22	F.	Gall bladder disease; very slow clot formation.	10.5
23	M.	Leukemia; no organized clot formation; daily urinary Ca excretion, 0.015 gm.	9.8
24	"	Pneumonia, lobar; low cholesterol in serum.	8.7
25	"	Diabetes; plasma Ca, 10.2, urine Ca normal.	10.1
26	F.	Acute anterior poliomyelitis; 6 years of age.	9.3
27	M.	Tuberculosis of the kidney.	10.3
28	"	" pulmonary.	10.6
29	"	Sciatica.	9.6
30	F.	Constipation, chronic.	9.8

TABLE II.

Calcium Content of the Serum in Nephritis.

No.	Sex.		Serum.	Urine (daily excretion).				
			Ca (mg. per 100 cc.).	Volume.	Total acidity.	pH.	Ammonia.	Ca.
				cc.			*gm.*	*mg.*
1	M.	Nephritis, chronic, mild; plasma Ca, 9.5.	9.4	420	124	5.9	0.08	8
2	"	Nephritis, chronic, mild; plasma Ca, 9.1.	9.4	1,750	Alk.		0.40	90
3	"	Nephritis, chronic, mild; plasma Ca, 9.2.	9.5	1,625	195	6.8	0.46	31
4	"	Nephritis, chronic; plasma Ca, 9.3.	9.4	2,040	482	6.9	1.12	220
5	"	" cardiorenal.	9.7					
6	"	" acute, severe.	10.2					
7	"	" " "	9.7					
8	F.	" cardiorenal.	8.9					
9	M.	" uremic; whole blood Ca, 5.9.						
10	F.	" chronic.	9.2					
		8 days later, no improvement.	9.3					
11	"	Nephritis, severe, acute.	7.1					
12	M.	Uremia, comatose.	6.8					
13	"	" plasma Ca, 6.9.	7.1	980	129	6.7	0.33	8
14	"	Nephritis, severe, acute; plasma Ca, 7.6.	7.8	780	312	6.8	0.52	6
		4 weeks later, improved.	8.6	1,300	281	6.7	0.39	18
		After 2 days calcium lactate (5 gm. per day).	9.0	1,400	282	6.8	0.38	20
15	F.	Uremia, comatose; non-protein N, 47 mg.	8.1	460	178	6.2	1.00	9
		3 days later, after 1 day calcium lactate (5 gm. per day); improved.	8.7	240		6.3	0.17	6
		After 2 days' treatment.		750	268	6.3	0.32	44
		" 3 " "	9.2	455	200	6.1	0.72	20
		2 days after treatment ceased.		201	67	6.0	0.14	13
16	"	Uremia, comatose; plasma Ca, 8.0; some urine lost.	8.2	150	86	6.5	0.16	20
		5 days later, after 1 day's treatment (5 gm. Ca lactate).		325	88	6.6	0.44	43
		After 2 day's treatment.	10.1	131	55	6.7	0.21	15

TABLE II—*Concluded.*

No.	Sex.		Serum.	Urine (daily excretion).				
			Ca (mg. per 100 cc.).	Volume.	Total acidity.	pH.	Ammonia.	Ca.
				cc.			gm.	mg.
17	M.	Nephritis, cardiorenal.		1,900	137	6.9		64
		1 day later, no treatment.	8.4	1,800	89	7.2	0.07	35
		After 1 day calcium lactate (5 gm.); marked edema.		920	74	7.2	0.69	28
		After 2 days' treatment.		350	41	7.1	0.16	10
		" 3 " "	9.6	480	111	7.1	0.44	10
		2 days after treatment ceased.		655	162	6.8	0.64	28
		5 " " " "		405	191	6.4	0.26	17
18	"	Nephritis, cardiorenal.	8.1	305	145	6.7	0.49	42
		2 days later after 1 day's treatment (5 gm. Ca lactate).		650	267	6.7	1.03	133
		After 2 days' treatment.		620	191	6.7	0.21	90
		" 3 " "	7.9	400	204	6.7	0.25	53
		5 days after treatment ceased.		600	121	6.8	0.43	32
19	"	Nephritis, cardiorenal with marked edema; urinary albumin 2.8 parts per thousand.	10.2	1,000	190	6.7	0.01	33
		1 day later after 1 day's treatment (5 gm. Ca lactate).		800	225	6.7	0.05	35
		After 2 days' treatment, improved.		1,100	163	6.7	0.02	39
		" 3 " " albumin 1.7 parts per thousand.	10.4	900	122	6.7	0.01	39
		1 day later, catharties only.		600	168	6.8	0.04	22
		2 days " no treatment.		910	290	6.6		28
20	"	Edema due to abdominal neoplasm.		300	147	6.8	0.52	4
		1 day later.	8.9	290	102	6.8	0.56	3
		2 days later after 1 day's treatment (5 gm. Ca lactate).		435	127	6.8	0.65	5
		After 2 days' treatment.		410	123	6.7	0.48	3
		" 3 " "	9.0	550	113	7.1	0.59	5
		3 days after treatment ceased.		438	118	6.7	0.39	3
		5 " " " "		250	75	6.6	0.27	2
21	F.	Pernicious vomiting of pregnancy.	10.1	1,070	545	6.7	2.30	161
		1 day later after sodium bicarbonate administration.		460	163	6.9	1.19	14
		2 days later, able to leave hospital.		450	168	6.9	0.28	12

The most obvious conclusion from these data is that in uremia the serum calcium values are nearly always low. In this condition low values for calcium have been shown to occur by Marriott and Howland (4) who have correlated these findings with high inorganic phosphate of the serum and the existence of an acidosis which may be secondary to the increase in phosphate, the latter probably also accounting for the decrease in blood calcium. While this decrease in serum calcium is notable as contrasted with findings in most other conditions, the fact that values of less than 8.0 mg. are rare, even where the condition approaches coma, rather leads us to emphasize the great constancy of this element as compared with most blood constituents. *A priori* this might appear remarkable in view of the insolubility of the carbonates and phosphates of calcium and consequent difficulties of excretion and metabolism. On the other hand it appears that the plasma is a saturated or supersaturated solution of calcium bicarbonate. Hence any appreciable or permanent increases are not readily brought about, while at the same time the calcium present is not readily reduced in amount by precipitation, nor by excretion on account of the large reserves of calcium in the bones. Thus in nephritis notable decreases in calcium appear to occur only in the most severe types where we know the blood composition to be in other respects markedly altered. In mild or chronic nephritis or cases mainly cardiac in character the calcium values were normal or but little below normal. In uremia comatose in character values of 7.0 to 8.0 were usually obtained, the lowest value being 6.8.

The composition of the urines as far as acidity and ammonia are concerned serve mainly to emphasize the fact that the acidoses of uremia are due mainly to retention and very little to overproduction of acid. In fact in many cases the urinary acidities and ammonia outputs were subnormal rather than otherwise. This supports also the view of Marriott and Howland that the acidosis is due to acid phosphate which has but little tendency to withdraw ammonia from the tissues.

The urinary calcium excretion in nephritis, particularly in the severe types, was usually very low. Thus in Cases 13, 14, and 15 (Table II) only about 10 mg. of calcium were excreted per day as compared with average normal excretions of 100 to 300

mg. Clearly the low serum values cannot be attributed to urinary losses of calcium. It would rather appear that the low plasma calcium affects excretion unfavorably, while there appears also to be a lowered capacity of the kidney cells to excrete calcium. Inasmuch as calcium appears to be excreted by these cells as an acid phosphate the same disturbance in cellular metabolism would account for the retention of both phosphate and calcium.

The influence of calcium lactate in amounts of 5 gm. per day was studied in several cases of nephritis. In four cases of severe uremia perceptible increases toward the normal in serum calcium were noted with some simultaneous improvement in clinical condition. It must however be borne in mind that similar increases in serum calcium were found where improvement occured without calcium administration. In two cases no appreciable increase in serum calcium was noted but in one of these the calcium level was normal to begin with. In no cases after the administration of these large amounts of calcium were abnormally high serum values obtained. This agrees with findings by us in tuberculosis.

In most cases calcium administration led to relative increases in urinary calcium excretion, although the excretions remained low, very markedly so indeed as compared with the calcium ingestion. The influence appeared greatest in cases where urinary acidities and ammonia values were highest. On the other hand in Case 19 (Table II) where the nephritis was less severe but the edema more generalized and the ammonia excretion was extremely low calcium administration had no effect upon the excretion of ammonia or of calcium itself.

Some light is thrown on these findings by the observations made on Cases 20 and 21 (Table II) which were non-nephritic in character. The first of these was a case of edema due to an abdominal neoplasm. Here the serum calcium level was but little below normal and was unaffected by calcium administration. Only 2 to 5 mg. of calcium were excreted daily by way of the urine and this excretion was entirely uninfluenced by the administration of large amounts of calcium salts.

Quite different were the findings in pernicious vomiting of pregnancy. Here although serum calcium was normal the ex-

cretion of calcium, ammonia, and acid was very high. After alkali administration there was an immediate drop in calcium excretion to less than 10 per cent of the original value. Similar rapid decreases in acidity and ammonia were noted, and by the 4th day the patient had nearly recovered. Apparently calcium excretion increased with an excessive acid production, secondarily to the heavy drain on the alkali metal reserve which is more readily mobilized. When alkali was given the calcium excretion became at least temporarily subnormal.

SUMMARY AND CONCLUSIONS.

The calcium content of human blood serum was determined in several normal cases and in a number of pathological conditions. In the normal cases values lying between 9 and 11 mg. of calcium per 100 cc. were obtained. In nearly all of the pathological conditions studied, including cases where the blood clotted with extreme slowness, a similar range was observed, indicating a great constancy of this element in the blood serum. Distinct decreases were noted in cases of hematogenous jaundice, eclampsia, pneumonia, and particularly uremia. In several cases of uremia increases in serum calcium were noted on improvement in the clinical condition and following administration of calcium lactate. The urinary calcium excretion in severe nephritis was found to be low and calcium lactate administration brought about but slight absolute increases. Where marked general edema occurred, with or without nephritis, the excretion of calcium was unaffected by increased ingestion. In a case of pernicious vomiting of pregnancy with severe acidosis, alkali administration decreased calcium excretion to 8 per cent of its original value.

It is pointed out that calcium determinations on whole blood are of little value unless the relative volume of plasma and corpuscles is known.

The authors desire to express their obligation to Dr. Philip B. Hawk for the opportunity of carrying out this work and for suggestions.

BIBLIOGRAPHY.

1. Lyman, H., *J. Biol. Chem.*, 1917, xxx, 1.
2. Halverson, J. O., Mohler, H. K., and Bergeim, O., *J. Am. Med. Assn.*, 1917, lxviii, 1309.
3. Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.
4. Marriott, W. M., and Howland, J., *Arch. Int. Med.*, 1916, xviii, 708.

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

II. THE MINIMUM REQUIREMENTS OF THE TWO UNIDENTIFIED DIETARY FACTORS FOR MAINTENANCE AS CONTRASTED WITH GROWTH.

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In a former paper from this laboratory McCollum and Pitz (1) have discussed the etiology of experimental scurvy in the guinea pig and its bearing on the "vitamine" hypothesis of Funk, which postulates the existence of a number of unidentified dietary essentials each of which acts as a protective substance. According to this hypothesis the absence of one or another of these leads to the development of a specific syndrome; in one case scurvy, in another beri-beri, in another rickets, and in still another pellagra. McCollum and Pitz demonstrated that scurvy in the guinea pig could not possibly be a "deficiency disease" in the sense in which Funk and his school employed this term. In other papers McCollum, Simmonds, and Pitz (2) have made it clear that the several symptom complexes recognized as being due to faulty diet can easily be accounted for by unsatisfactory relationships among the well recognized constituents of the normal diet. We have therefore, in the light of a great amount of systematic study of simplified diets, been forced to accept the view that among the diseases enumerated the "vitamine" hypothesis is a satisfactory explanation for polyneuritis only, and that of the four this is the only "deficiency disease" in the sense intended by Funk.

That another "deficiency disease" is possible and sometimes occurs there can be little doubt. McCollum and Davis (3)

first pointed out that certain mixtures of fats of animal origin, as butter fat, egg fat, and the fats extracted from the internal organs, *e.g.*, the kidney, liver (4), etc., contain something which is absolutely indispensable for either maintenance or growth, and that this substance is not found in vegetable oils or fats and in but very small and inadequate amounts in the body fats of animals. It is present in abundance in the leaves of plants and in a very few seeds, conspicuous among the latter being flaxseed and millet seed. We have shown how when the diet is inadequate in its content of this substance which we designate fat-soluble A, the animals become emaciated and suffer from edema of the eyes. Blindness results if the animals are permitted to go without this dietary essential or with an inadequate supply for a sufficient time. We have many times rescued animals from the threshold of death by the administration of one of these growth-promoting fats when nutrition had failed because the diet contained fats from vegetable sources only and no other source of the fat-soluble A.

In 1904 Mori (5) in Japan first called attention to what he believed to be fat starvation in infants which he observed in times of famine, and described eye troubles suggestive of those which we have found to result from this specific dietary deficiency. He pointed out the efficiency of chicken liver and of eel fat as curative agents. Mori observed over 1,400 cases in children of 2 to 5 years affected with xerosis of the conjuctiva and keratomalacia terminating often in blindness. Mori says the disease does not occur among fisher folk, but among peoples whose diets are principally of vegetable origin. This is significant in determining its cause. Bloch (6) has recently described severe cases of necrosis of the cornea with ulceration in forty cases at Copenhagen. The children had been fed nearly fat-free separator skim milk, and were atrophic or dystrophic and anemic. Bloch attributed the disorder to "fat starvation," since the children responded to breast milk feeding, or, in the case of older ones, to whole milk mixtures and to cod liver oil administration. Czerny and Keller (7) describe a similar condition of the eyes in children suffering from malnutrition as the result of being restricted to a cereal diet.

We feel confident that these cases of xerophthalmia reported by Mori and Bloch should be looked upon as a "deficiency disease" not hitherto recognized in its true relation to diet. It is not, as these authors believe, a "fat starvation" which produces the condition, but a lack of the unidentified dietary factor, fat-soluble A, which occurs in just those foodstuffs which they observed to possess curative properties. Since we are in possession of a method for producing an experimental xerophthalmia, we plan in the immediate future a thorough study of this condition.

There are then, we believe, two deficiency diseases in the sense in which Funk employed this term. One of these is polyneuritis which Funk recognized. This is due to a lack of an adequate supply of the unidentified water-soluble B which is abundant in all natural foodstuffs but is practically absent from purified starch, polished rice, sugars, and all fats from both animal and vegetable origin. The other we believe is the syndrome described above in which the two most prominent features are emaciation and xerophthalmia. Scurvy, pellagra, and rickets we believe can be accounted for by errors in the diet without assuming a protective substance for each of them.

Pellagra we shall discuss from the etiological standpoint in a forthcoming paper.

We have considered the diseases of dietary origin in the present paper only to present the main reasons why we hold the view that there are in reality two and only two unidentified dietary essentials in addition to the well recognized factors, proteins, carbohydrates, fats, and inorganic salts. We have secured chemical evidence which lends strong support to the idea that what we term water-soluble B is in reality but a single substance rather than a mixture containing several physiologically indispensable complexes. This phase of the problem we shall discuss under a separate title in the immediate future.

The object which we had in view in collecting the data presented in this paper will be briefly explained. It is well known that an animal during the growing period may be fed a diet adequate in all respects except that it contains too small an amount of protein of the quality contained in the food to support growth, yet enough to maintain it without loss of body weight over a long

period (8). The same idea may be expressed in the possibility of suspending growth because of a shortage of a single amino-acid in the diet. The work of Osborne and Mendel (9) gave the impression to several investigators that they (Osborne and Mendel) had discovered in lysine, an amino-acid which was necessary for growth but unnecessary for the processes of repair of tissue wasted in endogenous metabolism. The evidence rested upon experiments in which gliadin supplied 93 per cent and "protein-free milk" 7 per cent of the nitrogen of the diet and successful maintenance, but inability to grow was demonstrated. Gliadin was at that time believed to be free from lysine, hence the above conclusion. Osborne, Van Slyke, Leavenworth, and Vinograd (10) have since shown that the most carefully purified gliadin yields about 0.75 per cent of lysine, so the premise for the entire line of reasoning regarding the peculiar rôle of this amino-acid became untenable.

Sweet, Corson-White, and Saxon (11) at once attempted to make use of this supposedly established principle to control the growth of tumors.. If the diet of Osborne and Mendel did not support growth, would a neoplasm, which is analogous in many respects to embryonic tissue be able to grow upon a host receiving such a food supply? While their results seemed to substantiate the view that a tumor is not able to grow so well on a host restricted to the experimental diet, the data lose somewhat in interest since the theory on which the work was done has been found to be based upon erroneous reasoning.

The protein is evidently not the most satisfactory dietary factor upon which to attempt to differentiate between the conditions essential for maintenance as contrasted with growth. Van Slyke and Meyer (12) have shown that even during a prolonged fast the normal content of amino-acids is maintained in the blood and muscles through autolysis of the muscle proteins. This fact alone would make it improbable that such an experiment as that of Sweet, Corson-White, and Saxon should succeed. Van Slyke and Meyer's studies revealed a factor of safety of the greatest importance in the mechanism whereby during starvation the blood always contains a suitable pabulum to support the nutrition of the nervous system, internal organs, and heart even at the expense of great wastage of the skeletal muscles.

Another line of attack of the problem of controlling tumor growth has recently been tested by Benedict and Rahe (13) through such an adjustment of the diet as would insure maintenance of the adult tissues of the host, but make growth impossible. They sought such an adjustment in limiting the experimental animals to a diet adequate in all respects except for its content of "vitamine." The latter they supplied by small additions of yeast to the food. These investigators hoped to determine whether the normal adult tissues or the tumor tissue would be able to appropriate the supply of "vitamine" when the latter is present in the food in amount just sufficient to support life without loss of body weight. Their results indicate that the tumor cell can under these circumstances draw a supply of the water-soluble B from the blood stream even under conditions which require retrogressive changes in the normal body tissues.

Our charts give the results of a series of experiments designed to show the magnitude of the interval between the requirements of young rats for each of the unidentified dietary factors, fat-soluble A and water-soluble B, for maintenance as contrasted with growth. For comparison a series of tests with full-grown rats is described which, it was hoped, would give a clue to whether the adult tissues could maintain themselves on a supply of one or both of these dietary essentials lower than that required by the young for maintenance or growth.

We selected wheat germ as a source of the water-soluble B and employed butter fat as the source of the fat-soluble A. The diet employed was known to be satisfactory for normal growth and prolonged maintenance of health when an adequate supply of both of the unidentified essentials was furnished. *It consisted of casein 18.0, dextrin 76.3, salt mixture (185) 3.7, and agar-agar 2.0, (Chart 1). This was fed with (a) an abundance of fat-soluble A (as butter fat), (b) of water-soluble B (in wheat germ), and (c) with varying amounts, but always below the optimum requirements, of both wheat germ and butter fat as carriers of these unknown substances.*

These experiments it was hoped would give an answer to several questions of physiological interest.

1. Assuming that each of the food products, butter fat and wheat germ, are of constant quality what is the lowest intake of

each which can supply enough of the A or B respectively to just prevent loss of weight? This assumes, as has been previously shown and is illustrated anew by Chart 1, that an abundance of either unidentified factor without the other will not prevent rapid decline.

With our purified food mixture combined with wheat germ 1 per cent and butter fat 1 per cent, one adult rat has lived more than 13 months. It was not satisfactorily nourished on this diet, and for want of enough of the unknown A and B nearly all rats confined to such a ration may be expected to die within a few months (Chart 2, Lot 662). In this case the shortage of the factor A was greater than for the B. Our results indicate that there is no low plane of intake of either of these substances which can be said to maintain an animal without loss of vitality. When the minimal amount necessary for the prevention of loss of weight is approached the life of the animal is jeopardized if the diet is persisted in.

2. When both essentials A and B are supplied in amounts just above the maintenance needs, will growth be proportional to the supply of these, or must a certain excess of each over this minimum be present before growth in the young can take place?

Charts 4, 5, and 6 indicate that within certain limits growth is proportional to the supply of the fat-soluble A and water-soluble B in the diet, all other factors being properly adjusted. Failure of some to grow where others do so indicates that, even with our stock of animals which has been carefully selected for years by elimination of unfit adults from the breeding stock and the selection of the finest specimens for breeding, uniformity of performance under nutritive conditions adverse in these specific ways is not to be expected.

3. Is the requirement of either A or B for maintenance or growth less when the other of the two unknowns is present in liberal amounts, than when both are supplied in amounts near the minimum?

The answer to this question is definite and clearly demonstrated by our observations in many experiments in which two or more dietary factors fall below the optimum in quality or quantity. We have previously described rations derived from wheat germ with purified food additions, with which for a few

weeks normal growth may be attained, followed later by depression of growth with permanent stunting; or in certain cases full adult size may be attained but failure partial or complete in reproduction or rearing of the young has been observed. The improvement of a single dietary factor, which may be protein, inorganic content, or fat-soluble A, can enable animals on these same diets to perform their functions in a nearly normal manner (14). A quantity of either the fat-soluble A or the water-soluble B which may be just sufficient when all other dietary factors are of satisfactory quality, will not, when a second factor is less well constituted, induce well-being in the same degree as when a more generous supply is furnished. The animal can tolerate being limited to a very low intake of either the dietary A or B much better with an otherwise excellent diet than when it is less well constituted. (Compare Lots 663 and 665, Chart 3.)

4. What is the effect on the health of animals, of limiting them to a minimum supply of either the dietary A or B the remaining one being supplied in abundance; or of limiting the supply of both to near the minimal requirements? The answer to this question we expected to obtain by observing whether as the actual minimum for each of the two substances was approached the mortality of the animals should greatly increase while a few were able to maintain body weight and the appearance of health over a long period.

Without exception the records shown in the charts accompanying this paper indicate that it is a dangerous procedure to attempt to fast an individual selectively for one or both of these dietary essentials. We believe therefore that efforts directed toward the control of tumor growth by this means can never become of practical value. The life of the host will be cut short if the experimental conditions imposed are sufficiently rigid to render growth impossible.

The death of the animals, whose curves are marked P, was preceded by loss of muscular control. From the similarity of the behavior of these rats and of pigeons which are brought into a pathological state from a diet of polished rice, we believe that the rats exhibit the typical symptoms of polyneuritis.

Credit is due to Mr. W. Pitz for assistance in the preparation of materials employed in this work.

BIBLIOGRAPHY.

1. McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.
2. McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333.
3. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167.
   McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 361.
   Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xvi, 423.
4. Osborne and Mendel, *J. Biol. Chem.*, 1913, xvi, 423.
5. Mori, M., *Jahrb. Kinderheilk.*, 1904, lix, 175.
6. Bloch, C. E., *Ugeskruft for Laeger*, 1917, lxxix, 349. Cited from *J. Am. Med. Assn.*, 1917, lxviii, 1516.
7. Czerny, A., and Keller, A., Des Kindes, Leipsic, 1906, pt. 2, 67.
8. McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 415.
   Osborne and Mendel, *ibid.*, 351.
9. Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325, 334.
10. Osborne, T. B., Van Slyke, D.D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.
11. Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *J. Biol. Chem.*, 1915, xxi, 314.
12. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913–14, xvi, 197.
13. Benedict, S. R., and Rahe, A. H., *J. Cancer Research*, 1917, ii, 159.
14. McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 340.
15. McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.
16. McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 211.

CHARTS.

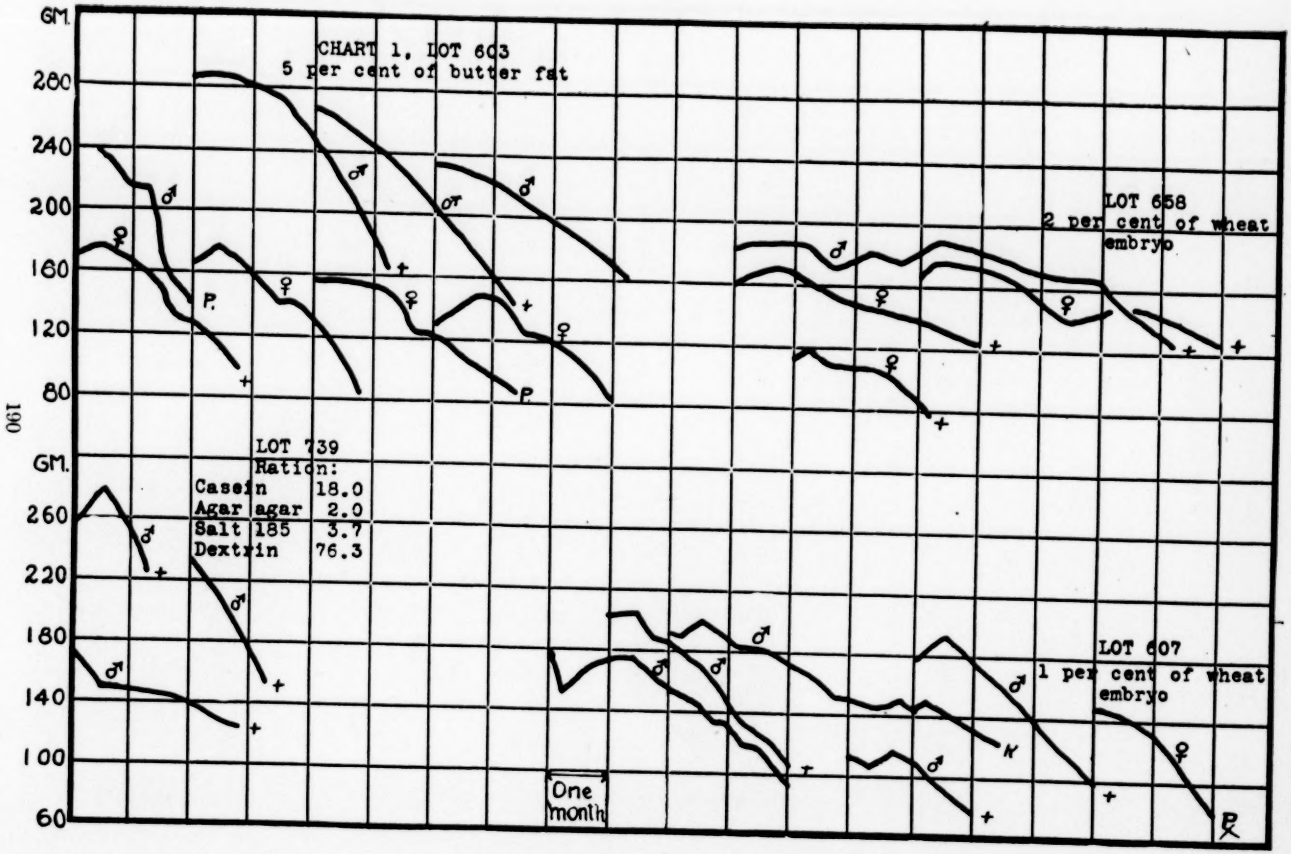

GM.
280
240
200
160
120
80
CHART 1. LOT 603
5 per cent of butter fat
LOT 658
2 per cent of wheat embryo
P.
P
GM.
260
220
180
140
100
60
LOT 739
Ration:
Casein 18.0
Agar agar 2.0
Salt 185 3.7
Dextrin 76.3
LOT 607
1 per cent of wheat embryo
One month
P

CHART 1. Lot 739. These curves illustrate the rate of decline in body weight of adult rats fed a diet which was adequate in protein, carbohydrate, and inorganic salts, but lacking both the unidentified dietary factors, fat-soluble A and water-soluble B.

Lot 603 received the same food as Lot 739 but with 5 per cent of butter fat to furnish the fat-soluble A. Without the second unknown essential factor B, loss of weight is uninterrupted and death follows in from 60 to 100 days. 5 per cent of butter fat furnishes an abundance of the dietary A, as is shown by many instances in our records of animals which have grown from weaning time to normal maturity and brought forth and reared the normal number of young (four or five litters).

Lots 607 and 658 show that in adult rats about 1 year old and in good condition and capable under favorable conditions of living 2 years longer, the span of life is greatly shortened by limiting the animals to the content of unidentified dietary factors contained in 1 or 2 per cent of wheat germ. With 1 per cent of germ loss of weight was fairly rapid while with 2 per cent two died after 2 and 4 months, two others lived 5 and 7 months respectively and death supervened with but little loss of weight.

Wheat germ contains a small amount of the fat-soluble A (15) and a liberal amount of the water-soluble B. When supplemented with a suitable amount of butter fat (fat-soluble A) 2 per cent of wheat germ furnished enough of the factor B to support growth (Chart 5, Lot 838).

These records are of particular importance for the light which they throw upon the debilitating effects of greatly restricting the intake of either or both of the two unidentified dietary essentials. The prospects for controlling tumor growth through reducing the content of either of these substances in the diet are not favorable for such reductions are attended by great danger to the host.

Composition of salt mixture 185:

	per cent
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4.H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2.H_2O$	0.540
Fe citrate	0.118
Ca lactate	1.300

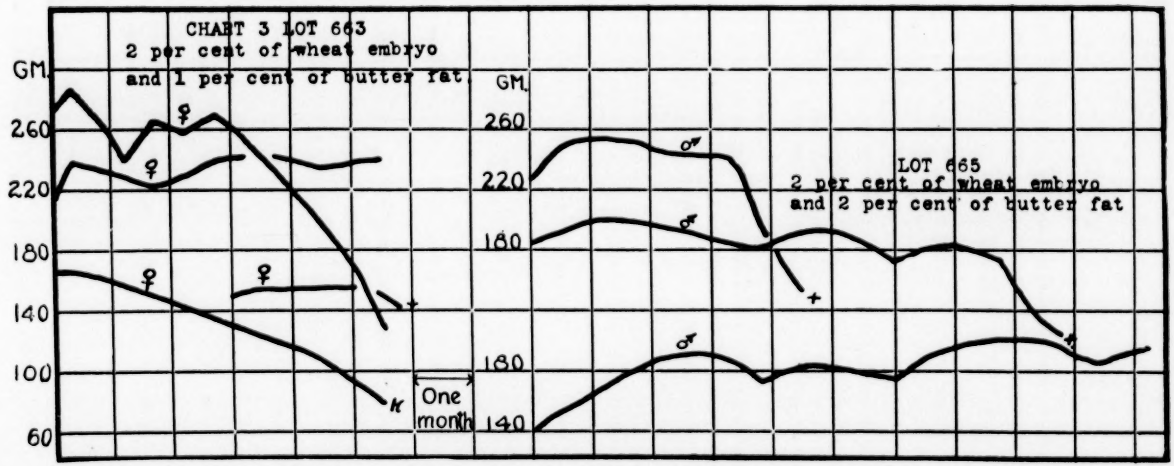


CHART 3. Lots 663 and 665 illustrate the fact that the minimal amount of butter fat which can supply the fat-soluble A in amount sufficient to maintain a vigorous adult animal in a state of health is not far from 1 per cent of the food mixture. 2 per cent of wheat germ suffices for the factor B, but in Lot 663 which received but 1 per cent of butter fat two steadily declined, while one was somewhat heavier after 5 months than at the beginning of the experiment. Subnormal vitality will quickly be revealed by a diet in which there is a shortage of either of the unidentified dietary essentials.

Lot 665 were noticeably benefited by having 2 per cent of butter fat, as compared with Lot 663 which received the same diet with but 1 per cent. It is better to have a liberal supply of one and a minimal supply of the other of the A and B than the minimal allowance of both.

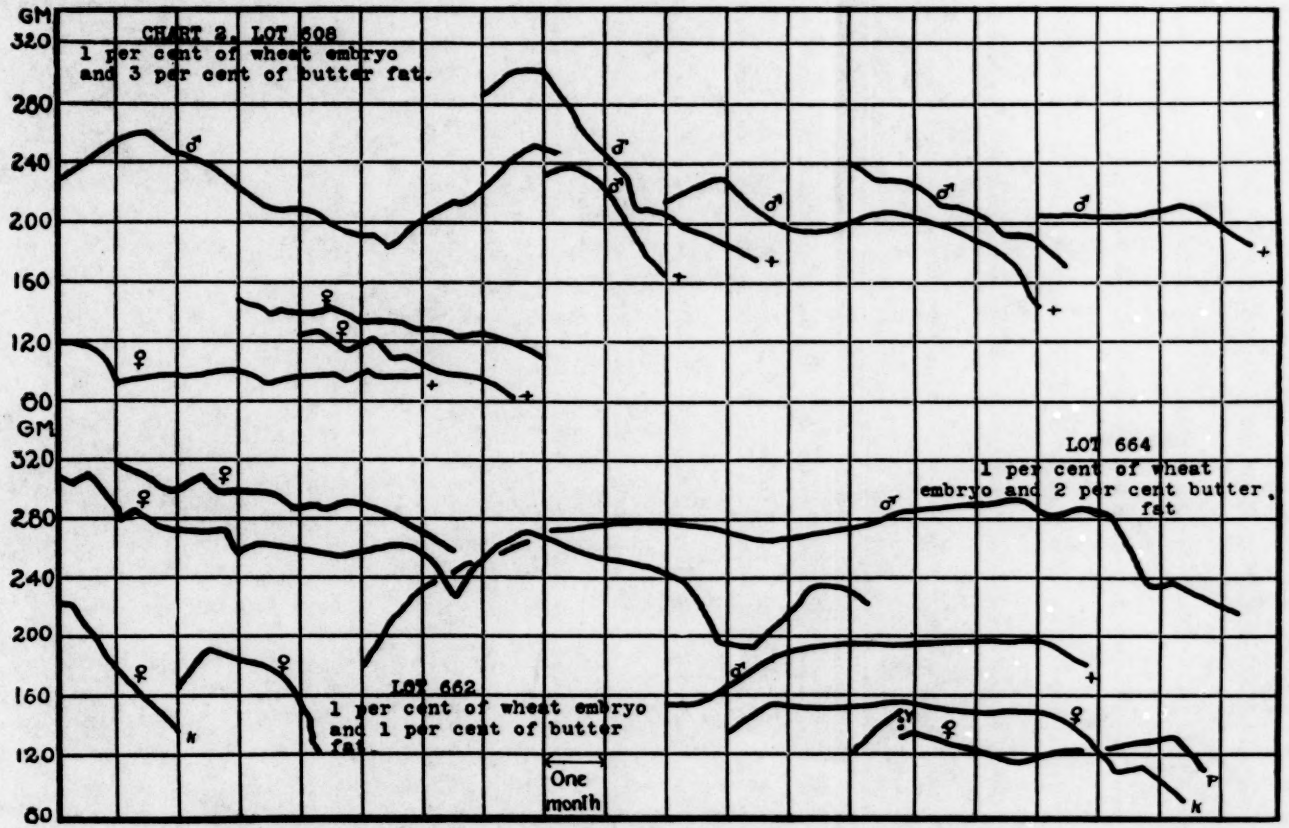


CHART 2. This chart illustrates the behavior of adult rats when fed purified foodstuffs as in Chart 1, but with the water-soluble B supplied by 1 per cent of wheat germ and the fat-soluble A by 1, 2, and 3 per cent of butter fat respectively in Lots 662, 664, and 608.

On the first of these (Lot 662) none of the rats were able to maintain their weight, but in one case a female lived 400 days. Two individuals were unable to tolerate the limitations of the diet and suffered rapid decline.

Lot 664 which received 1 per cent of wheat germ and 2 per cent of butter fat practically maintained their weight in all instances. One individual (male at top) lived 14½ months with a considerable gain in weight followed by decline. The other three, while not losing weight, had their lives greatly shortened by the dietary deficiencies. One which produced a litter of young, all of which died soon after birth, later suffered typical polyneuritis.

In Lot 608 which received its unidentified dietary factors from 1 per cent of wheat germ and 3 per cent of butter fat did not contain a single individual among the nine animals in the group which was able to tolerate the diet without distinct loss of vitality, and greatly shortened span of life. The danger of degenerative changes is very great when the content of the dietary essentials A or B is reduced to near the minimal requirement.

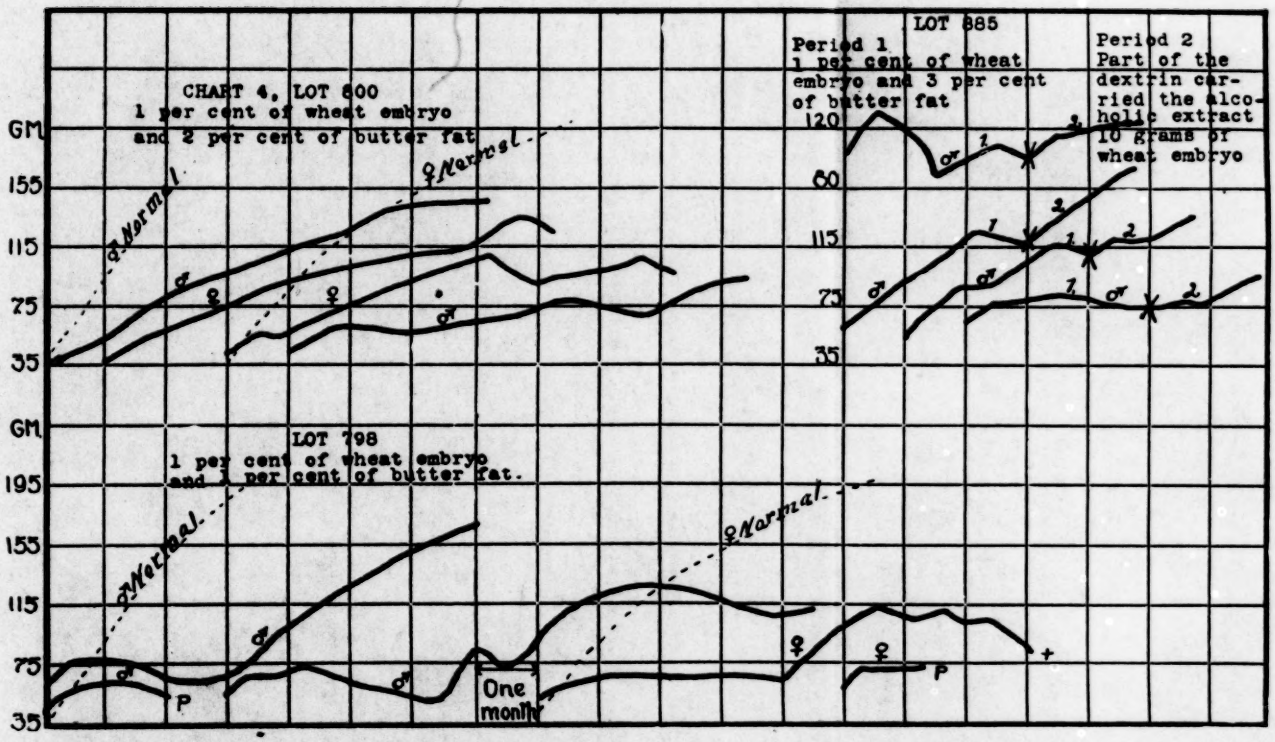


CHART 4. Lot 798 shows that a considerable amount of growth in young rats is possible on the amount of the dietary A and B in 1 per cent of butter fat and wheat germ respectively. It is a surprising fact that all suffered a period of suspension of growth at the beginning of the experiment, followed by a period of subnormal growth. This is not due to error in making up the ration, for it was made up anew at intervals of about 3 weeks during the entire experiment. Furthermore, the animals did not begin to grow at the same time.

Lots 800 and 885 illustrate the amount of growth which young rats are capable of making on a mixture in which 1 per cent of wheat germ furnishes the B and 2 and 3 per cent of butter fat the A. The young tolerated this diet better than one would expect from the behavior of adults on the same mixture (Chart 2, Lots 608 and 664).

In Period 2 of Lot 885 an alcoholic extract of wheat germ was added to the diet to supply more of the factor B. Although somewhat delayed there was a noticeable response in all individuals. One rat suffered two attacks of polyneuritis and was each time relieved within 48 hours by small doses of a water extract of wheat germ administered with a medicine dropper, since it was unable to stand.

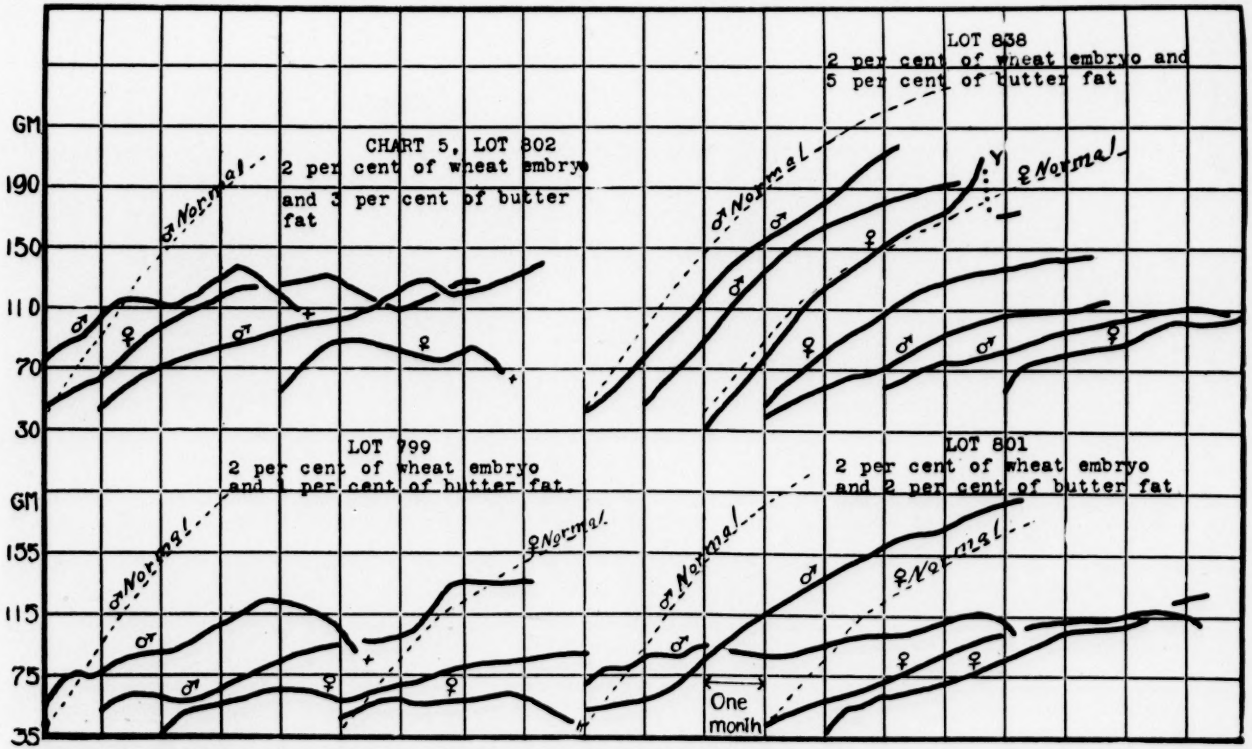


CHART 5. All groups received a ration of purified foodstuffs and the water-soluble B contained in 2 per cent of wheat embryo. Lots 799, 801, 802, and 838 show the stimulating action on growth of adding 1, 2, 3, and 5 per cent respectively of butter fat to the food mixture. On the latter food a single litter of young was born. These died within a few hours. The great difference in the vitality of individuals is shown by the failure of some to grow in Lot 838 where others were capable of doing so. The content of the factor B in 2 per cent of wheat germ is too low to induce optimum well-being.

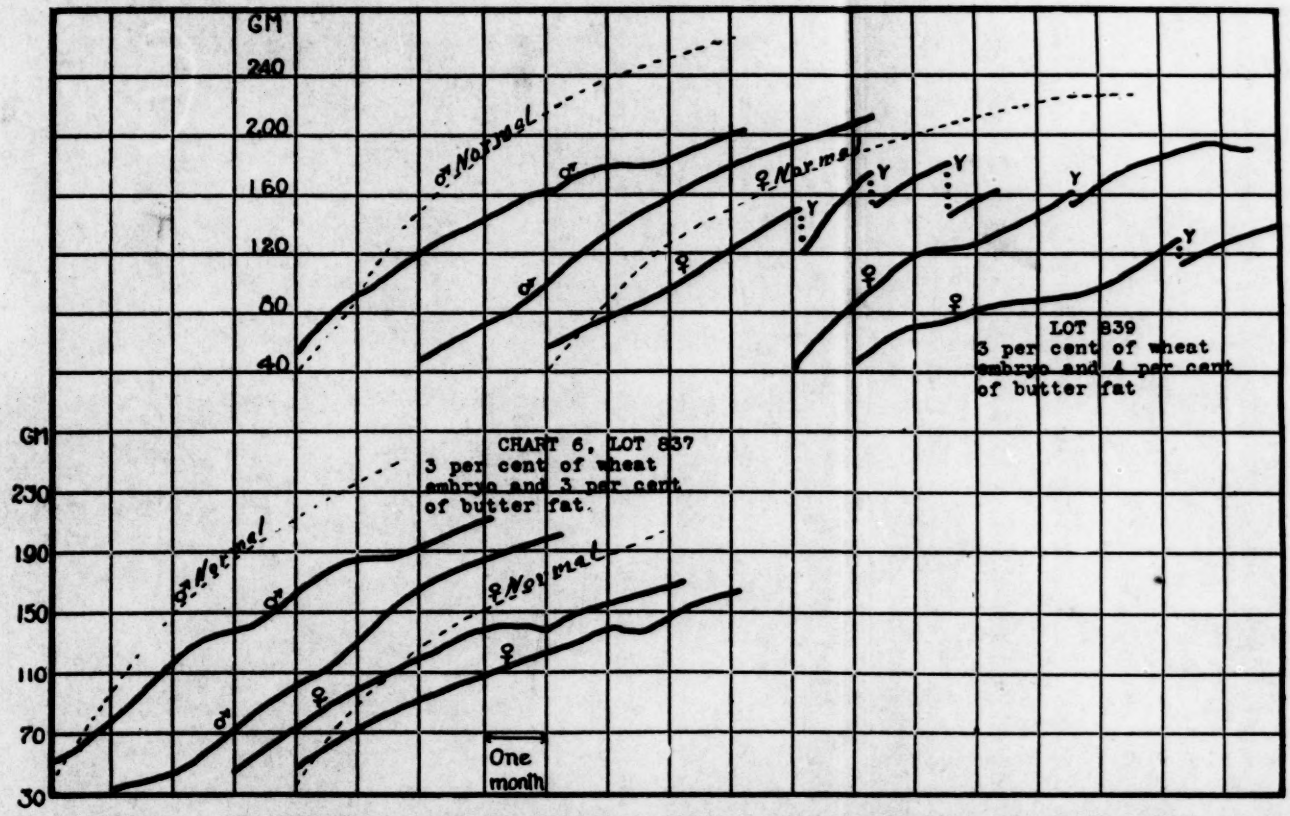
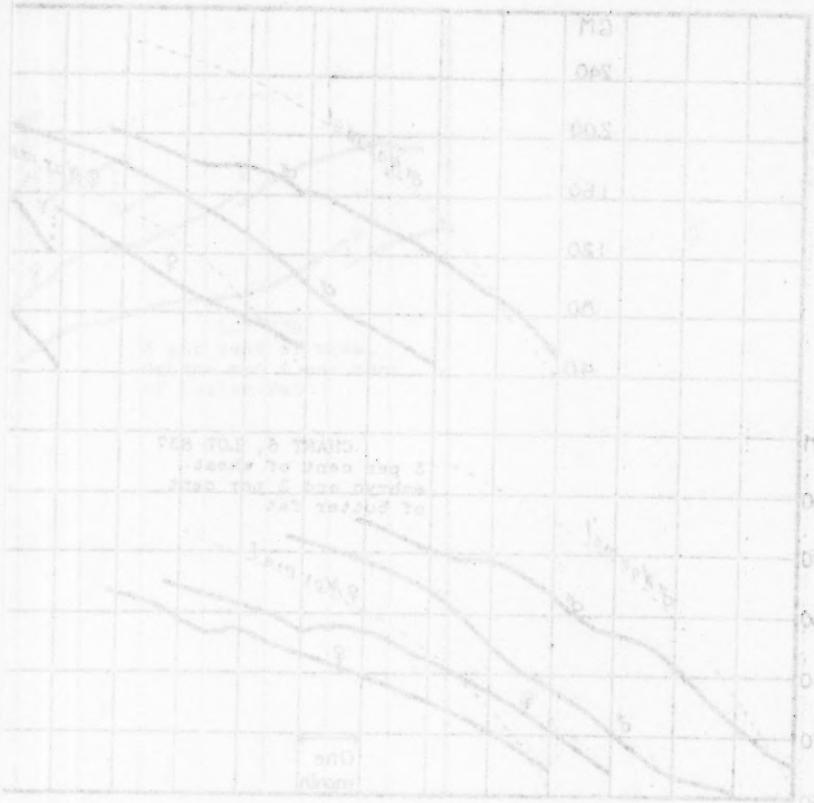


CHART 6. Lots 837 and 839 make clear the beneficial effects of having a *liberal* supply of the unidentified A and B in the diet. All the animals were undersized in both lots but in Lot 837 with 3 per cent of butter fat (fat-soluble A) two females failed to have any young, while in Lot 839 which made no better growth two females produced one litter each and a third three litters. None of these lived beyond a few days. The higher plane of butter fat improved the vigor of the rats as shown in their ability to produce young. They needed still more of the factors A and B to enable them to rear their young (16).

In the first paper of this series we pointed out that in our studies of mixtures of maize and bean proteins an amount of protein just sufficient to support growth at about the normal rate does not induce well-being in the degree that a moderate excess of protein over the actual requirement does.

Perhaps the data presented in this paper are not sufficient to justify a definite conclusion, but it appears that the young are more tolerant for a time than the adult of diets relatively deficient in the fat-soluble A and water-soluble B. The lack of uniformity of animals of apparent good vitality on such diets as we are describing emphasizes the probability that the vigor of the young may explain the results.

These data support the idea that for best results the diet should not approach the minimum requirements of any dietary factor.



THE PROBABLE ACCURACY, IN WHOLE BLOOD AND PLASMA, OF COLORIMETRIC DETERMINATIONS OF CREATININE AND CREATINE.

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Certain investigations, of which a preliminary account has already been published,[1] have required us to carry out within the last two years a large number of determinations of creatinine and creatine not only in whole blood, but also in plasma. The methods employed—those described by Folin,[2] and in slightly modified form by Myers and Fine[3]—were at first assumed to be in each of these situations equally and entirely reliable; but it soon became obvious to us that no final answer to the questions engaging our attention was possible until this assumption had been adequately tested. We were accordingly led to consider rather closely what degree of precision was actually attributable to the various determinations we were making.

Our first attempt[4] to reach a decision upon this point led us to the conclusion that, if the disturbing influence of the sodium picrate color be eliminated by the use, in the interpretation of the colorimeter readings, of empirically constructed standard curves, and if care be taken to protect the picric acid solutions employed from the deteriorating action of light, the determination of minute quantities of creatinine may be accomplished with a highly satisfactory degree of accuracy. Essentially the same conclusion was reached by Folin and Doisy,[5] who indicated a second method of minimizing the picrate effect, and demonstrated the frequent presence in fresh commercial picric acid of a chromogenic impurity similar to, if not identical with, the one which we had encountered only in old solutions. One positive result of these investigations was the vindication of the Folin method for creatinine against the severe strictures passed upon it by McCrudden and Sargent.[6] Unfortunately, as far as the

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[1] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1917, xxix, p. xviii.

[2] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

[3] Myers, V. C., and Fine, M. S., Chemical Composition of the Blood in Health and Disease, Cooperstown, N. Y., 1915, p. 19.

[4] Hunter and Campbell, *J. Biol. Chem.*, 1916–17, xxviii, 335.

[5] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

[6] McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxiv, 423.

positive demonstration of that method's accuracy was concerned, neither of them went further than the case of pure aqueous solutions. They did not suffice to prove the entire reliability of the determination in such a complex mixture as the blood. The reaction upon which the method is based is not a specific one; and we possess no guarantee that the blood does not normally contain, besides creatinine, other substances capable singly or collectively of simulating its behavior. Uncertainty upon this point affects not only the determination of preformed creatinine, but even more strongly that of creatine; for in the latter case one has to reckon with the possible development, as products of hydrolysis, of quite new reacting substances. It is a further complication that the case of whole blood in relation to these determinations is not necessarily the same as that of plasma; and there are occasions when it is important to know, if not the absolute, at least the relative accuracy with which creatinine or creatine can be determined in these different situations.

Considerations such as these imperatively suggested the need of proceeding beyond the case of pure solutions, and of attempting to ascertain as closely as possible the limitations in actual practice of every application of the colorimetric method for creatinine to blood analysis. The need was emphasized, as we proceeded, by the criticisms which from time to time have been directed against one or another of these applications by other workers in the field. Thus it has been maintained by Gettler[7] that the Folin method for the determination of blood creatinine, although capable on suitable modification of giving trustworthy results, is in its original form grossly inaccurate. More recently Wilson and Plass,[8] while conceding the correctness of that method as applied to plasma, have held that it exaggerates somewhat the true creatinine content of whole blood; and have expressed similar opinions regarding the relative value, in blood and plasma, of the Folin method for creatine. A lack of confidence in the latter is to be inferred also from a preliminary communication by Greenwald.[9] In our own examination of the subject we have of course found it necessary to take into account the views of each of the authors mentioned.

A decisive test of the accuracy of the colorimetric procedure, in any of its applications, might be furnished by the discovery of a really specific reaction, or by the quantitative isolation of the creatinine, preformed or total, in an identifiable combination. It is at present, and will probably long remain, impossible to check the method in any such manner. We have been forced, therefore, in endeavoring to reach a reasoned decision upon the

[7] Gettler, A. O., *J. Biol. Chem.*, 1917, xxix, 47.

[8] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413. Also Plass, E. D., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 137.

[9] Greenwald, I., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 115.

question, to rely upon evidence less direct in character. Such evidence we believe ourselves now to possess in sufficient abundance to warrant certain fairly definite conclusions. It has been obtained by the application of a variety of tests, which were based upon the following considerations.

1. When sodium hydroxide is added to pure creatinine in a saturated solution of picric acid, the reduction of the latter does not take place instantaneously but with a certain measurable velocity. If the amount of the colored reduction product be plotted against the time, the progress of the reaction will be graphically illustrated by a curve. This curve will have a characteristic form, and each time the Jaffé reaction is performed with pure creatinine under standard conditions the same type of curve will be reproduced. If the typical form be departed from, it can only mean that some other reaction, with a different velocity constant, is sharing in the development of the color.

2. Provided that colorimeter readings are interpreted, according to our practice, by reference to standard curves,[4] dilution of a pure creatinine solution will result in a strictly proportionate diminution of its colorimetrically estimated concentration; in other words, the final outcome of the determination will be independent of the dilution at which it is made. This is a simple consequence of the manner in which the standard curve is constructed. A mixture of creatinine with another similarly reacting substance would not necessarily obey this rule; for it might happen to exhibit a somewhat different relation between concentration and color intensity. If the determination, as made at one dilution, should fail to agree with that made at another, one would be justified in concluding that creatinine was not the only chromogenic substance present.

3. If a pure creatinine solution be compared with each of two standards, one of higher and one of lower concentration, the two determinations will give (within the ordinary limits of colorimetric work) identical results. This, like (2), follows from the structure of our interpretative curves. If the relation which insures this agreement be disturbed, and results be found to vary according to the standard employed, it will be evidence that at least a part of the total color owes its origin to something other than creatinine.

4. Applying these principles to the case of blood, we see that, if the color reaction there is due to creatinine alone, it must develop at such a rate as to yield a time curve identical with that of pure creatinine in the indicated concentration; the result of the determination must be independent of the extent to which the blood is diluted; and it must be a matter of indifference what particular concentration of creatinine is adopted as a convenient standard. The fulfillment of all these conditions will furnish not indeed absolute proof, but at least a strong presumption, that we are dealing with an unmodified creatinine reaction; the failure of any one of them will be conclusive evidence to the contrary.

From the points of view thus indicated we have examined the creatinine reaction as developed in picric acid filtrates from whole blood and plasma both before and after autoclaving. The methods for preformed and total creatinine were thus subjected to three distinct series of tests, the results of which will now be severally discussed.

The Rate of Color Development.

In order to study the rate of color development in creatinine solutions or blood filtrates the following procedure was adopted. A suitable standard[10] is placed in position in one of the colorimeter cups, and a watch indicating seconds is laid beside the instrument. The alkali is added to a conveniently large volume of the solution to be tested, and the time at which a complete mixture is effected is noted to within a couple of seconds. The solution is immediately placed in the second cup of the colorimeter, and the plunger is sunk until its side of the field is distinctly the lighter. The plunger is then carefully raised until the two sides just appear to have attained equality. The time is instantly noted, and the reading is taken at leisure. Again the plunger is depressed, and the whole proceeding repeated. In this way it is possible to make a succession of quite deliberate readings at the rate of at least one per minute. After 10 minutes

[10] In all the experiments of this section the fixed standard employed was one containing 0.2 mg. of creatinine in 100 cc. of saturated picric acid —a "1 mg." standard.

these readings are interrupted, but additional ones are taken at 15, 25, 40, and sometimes 60 minutes after the addition of the alkali. The creatinine values of the readings, ascertained by reference to our standard curves,[4] are plotted on squared paper against the times at which they were taken; and finally a smoothed curve is drawn through the points thus charted.

Crude as this method may appear, it is surprising how successfully, under favorable conditions, it can be operated, and how rarely any point is found to lie at more than a negligible distance from an ideal curve. Occasional irregularities were of course encountered, but never in such frequency or of such magnitude as to throw doubt upon the substantial fidelity of our curves to the actual course of each reaction.

Experiments have been made, and curves constructed, for each of the following cases:

1. A "1 mg. standard" of pure creatinine.
2. A "1.5 mg. standard" of pure creatinine.
3. A "2.5 mg. standard" of pure creatinine.
4. A standard corresponding to 1 mg. of creatinine with 100 mg. of glucose per 100 cc.
5. The filtrate from whole blood as obtained by the original Folin technique, in which saturated picric acid solution is added directly to the oxalated, but otherwise untreated blood.
6. The same filtrate after autoclaving.
7. The filtrate from whole blood treated according to the technique of Myers,[3] in which the blood is laked before being saturated with picric acid.
8. The filtrate from picric acid treatment of blood plasma.
9. The same after autoclaving.

The total number of curves constructed was forty-two, the minimum for any given case being two, and the maximum eleven. To tabulate the many separate observations from which these were derived, or even to reproduce individually all of the curves themselves, would be to take up a great deal of space without displaying very clearly their significance. The special characteristics of each group of curves can be more easily and more perspicuously illustrated by combining its members into a single composite curve. To effect this, all the curves of one category were first projected together upon the same chart, quantities of

creatinine being plotted as ordinates, time intervals as abscissæ. It was then noted at what points each of the abscissæ representing successive whole minutes cut the various curves. These points were translated into mg. of creatinine and averaged. The averages for every minute up to 10, and for the subsequent 15, 25, and 40 minute periods, were finally plotted upon a fresh chart, and joined to form a continuous curve.

The advantages of this procedure are obvious. It not only simplifies the presentation of our results, but nullifies, even more completely than is possible on the original curves, the influence of any accidental irregularity in the observations. The only possible objection to it is that it might impart to the results in any one group the appearance of a uniformity which they did not in reality possess. It deserves therefore to be expressly emphasized that no one of the original curves was incompatible with the conclusions drawn from the composites. The differences exhibited by the latter were found also in every single series of the former. Sometimes they were less striking, just as often more so; they always existed, and had always the same character. We are confident, therefore, that our method of presentation involves nothing more than the averaging of entirely consistent sets of results, and that, when the number of separate curves combined is large enough, the composite may be presumed to give a very accurate representation of the typical course of the reaction studied.

The curves themselves are shown on Charts I and II. The final averages from which they are drawn are recorded in Table I. The interpretation of the curves is facilitated by the calculation from these averages of the expression

$$k = \frac{1}{t} \log_e \frac{a}{a - x}$$

which is the velocity constant of the reaction on the assumption that it is a monomolecular one. The values found for k are likewise exhibited in Table I. It should be noted that they are calculated with reference to the first 10 minutes only, during which alone creatinine can be held responsible for developing the color.

TABLE I.

Average Velocity of Color Reaction in Different Cases.

x = apparent creatinine content at t minutes.
a = " " " " 10 "

$$k = \frac{1}{t} \log_e \frac{a}{a - x}$$

t	Pure creatinine.									Creatinine + glucose.			Plasma.			Whole blood.						Plasma autoclaved.			Whole blood autoclaved.		
	1 mg.			1.5 mg.			2.5 mg.									Folin.			Myers.								
	x	$a-x$	k	x	$a-x$	k	x	$a-x$	k	x	$a-x$	k	x	$a-x$	k	x	$a-x$	k	x	$a-x$	k	x	$a-x$	k	x	$a-x$	k
min.																											
1	0.38	0.62	0.48	0.56	0.94	0.47	0.92	1.58	0.46	0.54	0.49	0.74	0.53	0.53	0.70	0.94	0.63	0.91	1.13	0.71	0.95	1.65	0.63	1.29	1.85	0.97	1.07
2	0.62	0.38	0.48	0.93	0.57	0.48	1.51	0.99	0.46	0.75	0.28	0.65	0.71	0.35	0.55	1.11	0.46	0.62	1.29	0.55	0.60	1.88	0.40	0.87	2.17	0.65	0.74
3	0.76	0.24	0.48	1.10	0.40	0.44	1.86	0.64	0.45	0.87	0.16	0.62	0.82	0.24	0.50	1.24	0.33	0.52	1.38	0.46	0.46	2.01	0.27	0.71	2.37	0.45	0.61
4	0.84	0.16	0.46	1.23	0.27	0.43	2.10	0.40	0.46	0.93	0.10	0.58	0.90	0.16	0.47	1.32	0.25	0.46	1.45	0.39	0.39	2.08	0.20	0.61	2.53	0.29	0.57
5	0.90	0.10	0.46	1.32	0.18	0.42	2.22	0.28	0.44	0.96	0.07	0.54	0.95	0.11	0.46	1.40	0.17	0.45	1.52	0.32	0.35	2.14	0.14	0.56	2.64	0.18	0.55
6	0.94	0.06	0.47	1.39	0.11	0.44	2.32	0.18	0.44	0.98	0.05	0.50	0.99	0.07	0.46	1.45	0.12	0.43	1.59	0.25	0.33	2.19	0.09	0.54	2.72	0.10	0.56
7	0.97	0.03	0.50	1.45	0.05	0.48	2.38	0.12	0.43	1.01	0.02	0.56	1.02	0.04	0.44	1.49	0.08	0.43	1.66	0.18	0.33	2.23	0.05	0.55	2.77	0.05	0.58
8	0.99	0.01	0.58	1.48	0.02	0.53	2.43	0.07	0.45	1.02	0.01	0.58	1.04	0.02	0.49	1.51	0.06	0.41	1.72	0.12	0.34	2.25	0.03	0.54	2.80	0.02	0.62
9	1.00			1.49	0.01	0.56	2.47	0.03	0.49	1.02	0.01	0.52	1.05	0.01	0.52	1.54	0.03	0.44	1.78	0.06	0.38	2.27	0.01	0.60	2.82		
10	**1.00**			**1.50**			**2.50**			**1.03**			**1.06**			**1.57**			**1.84**			**2.28**			**2.82**		
15	1.00			1.50			2.50			1.05			1.09			1.67			2.19			2.33			2.85		
25	1.00			1.50			2.50			1.08			1.13			1.77			2.44			2.37			2.85		
40	1.00			1.50			2.50			1.11			1.17			1.84			2.80			2.44			2.88		

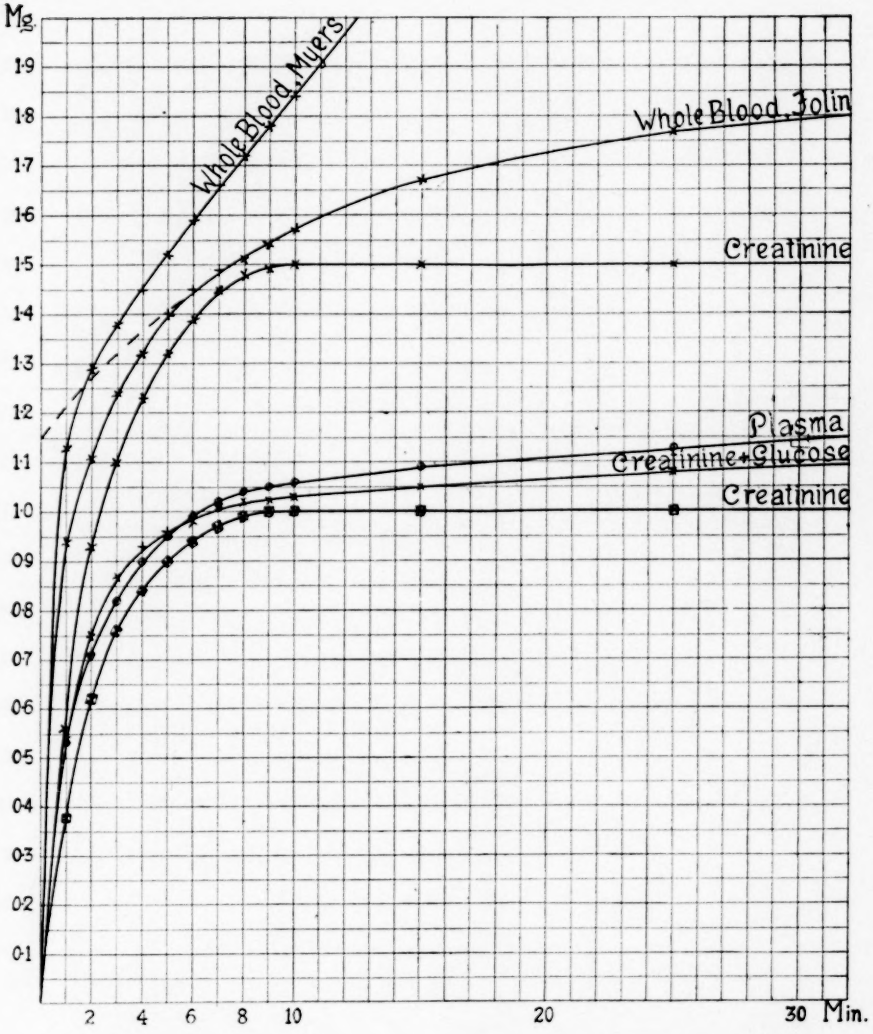

CHART I. Time curves for the reaction with creatinine, creatinine + glucose, plasma, whole blood (Folin's technique), and whole blood (Myers' technique).

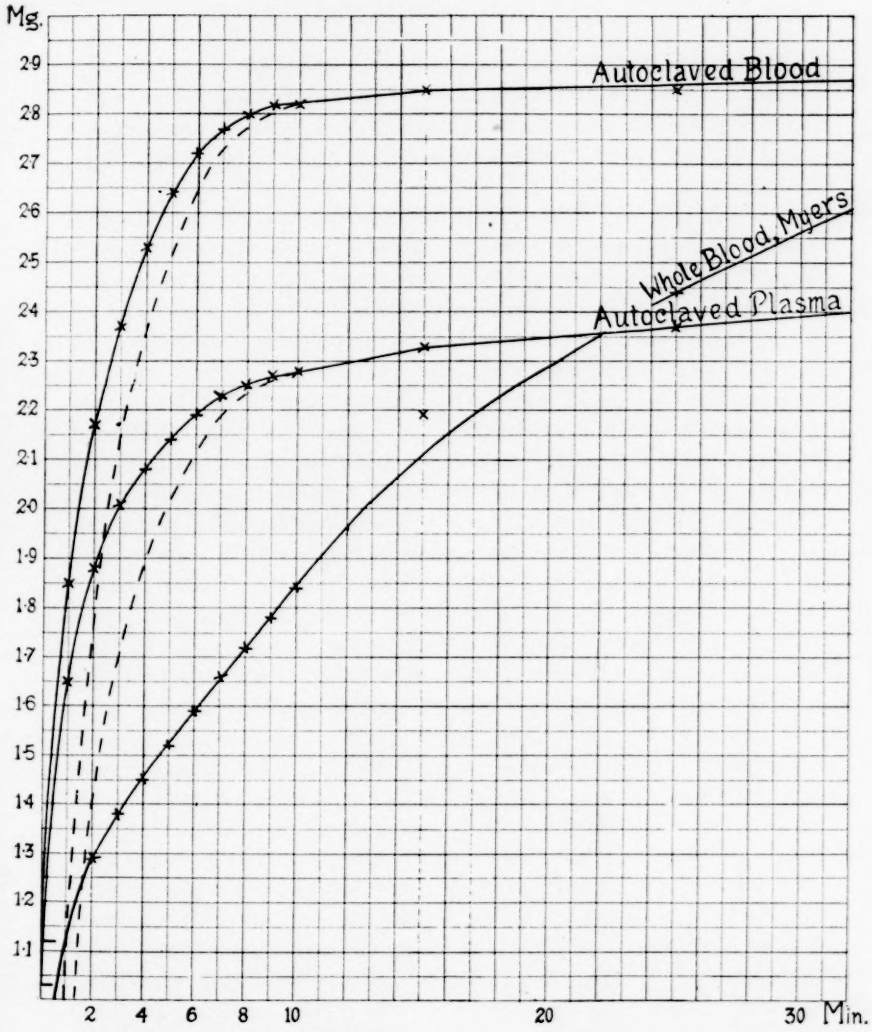

CHART II. Time curves for the reaction with autoclaved whole blood and autoclaved plasma; and continuation of the curve for whole blood (Myers' technique).

Discussion of the Curves.

A reference to the charts will show that the eight curves finally available for comparison do not all assume exactly the same form. A discussion of each in turn will serve to elucidate the consequences which, we believe, may be drawn from the differences presented.

Pure Creatinine.—The time curve shown in Chart I for the "1 mg." pure creatinine standard is a composite constructed from three single ones, which as a matter of fact almost exactly coincided with one another. Its form indicates that at any given instant the rate of increase of the color is proportional to the amount of creatinine which has still to react. This is made clear by the fact that the value of k as recorded in Table I is as nearly constant as the nature of the method used could lead one to expect. The only aberrant value is at the end of the curve; and this is a point where a comparatively small error of observation would have a large effect in the formula by which k is calculated. The evidence therefore bears out, better even than might have been anticipated, the assumption that the reaction is a monomolecular one. For all practical purposes the reaction may be regarded as complete, and the curve as having reached its summit, at the 9th minute after the addition of the alkali. After that point the curve becomes a horizontal line.

The conclusion that we are dealing with a reaction of the first order is confirmed by the curves for the 1.5 and 2.5 mg. standards, of which the former only is reproduced in the chart. These also conform closely to the logarithmic formula, reach their maxima about the 9th minute, and yield for k a practically constant value, approximately the same as with the first curve. Rejecting as certainly erroneous all values for k higher than 0.50, the averages at the three different concentrations are 0.48, 0.45, and 0.45. The true velocity constant of the creatinine reaction is probably not far from the grand average of 0.46. It may be pointed out that with the character of the reaction thus firmly established, it becomes permissible to calculate, on the basis of the recorded observations, the time curve for any given concentration of creatinine.

Creatinine and Glucose.—The reduction of picric acid by glu-

cose in hot alkaline solution is a familiar reaction, which forms the basis of a well known quantitative method for the estimation of sugar in blood. Gettler[7] has recently drawn attention to the fact that the same reaction takes place, although much more slowly, at ordinary temperatures; and he appears to regard it as one factor at least in that gross exaggeration of the true creatinine content of blood to which, as he claims, the original technique of Folin conducts. He is also, it would seem, inclined to make it responsible for the progressive deepening of color which is noticeable in blood creatinine determinations long after the expiration of the prescribed 10 minute interval. That it must have some effect in each of these directions is self-evident; but the curve exhibited in Chart I, a composite from four single ones, would hardly suggest that that effect is a considerable one. At the 10 minute point it is seen that glucose, in such concentration as might be encountered in the analysis of normal blood, has contributed to the total color no more, on the average, than the equivalent of 0.03 mg. of creatinine; and even after 40 minutes it has raised the apparent creatinine content of the mixture hardly more than 10 per cent. We believe that, as a matter of fact, Gettler's reported experiments with glucose (see his Experiment II, page 52) tend to give an exaggerated idea of the probable effect of that substance in normal blood. The mixtures with which these experiments were made contain, if we understand correctly the figures of his table, not such a concentration of glucose as might be expected in a normal blood filtrate, but twice as much.[11] Nevertheless, in view of the striking character of the results reported, we have thought it worth while to control the evidence of our time curve by some additional experiments. These consisted simply in ascertaining, by comparison with appropriate standards, the amount of color developed on adding the prescribed proportion of alkali[12] to various combinations of glucose and creatinine in saturated picric acid solu-

[11] For 6 mg. in 15 cc. of blood filtrate would be 6 mg. in 3 cc. of blood, or 0.2 per cent.

[12] It should perhaps be expressly stated that in all our work we have used the proportion of sodium hydroxide recommended by Folin (0.5 cc. of 10 per cent solution for 10 cc.). Gettler employs a greater concentration, namely, 1 cc. of 2 N NaOH to 10 cc.

tion. The combinations tested and the results obtained are shown in Table II.

TABLE II.

The Effect of Glucose on the Creatinine Determination.

Experiment.	Creatinine per 100 cc.*	Glucose per cc.*	Standard of comparison at 20 mm.	Colorimeter readings.			Apparent creatinine content.	
				10 min.	1 hr.	24 hrs.	10 min.	1 hr.
	mg.	*mg.*		*mm.*	*mm.*	*mm.*	*mg.*	*mg.*
1	—	1.0	Sodium picrate.	18.3	16.7			
2	—	2.0	" "	17.3	15.5			
3	—	3.0	" "	16.2	13.8			
4	—	4.0	" "	14.4	12.8			
5	1.0	—	1 mg. creatinine.	20.0	20.1	19.9	1.00	0.99
6	—	1.0	1 " "	50	42**	19.1 10.7		
7	1.0	1.0	1 " "	20.0	18.5	15.5 7.7	1.00	1.12
8	1.0	1.0	1 " "	19.7	17.9		1.02	1.18
9	1.0	1.0	1 " "	19.5	18.5		1.04	1.12
10	1.0	1.0	1 " "	19.6			1.03	
11	1.0	4.0	1 " "	17.5	15.1	1.0	1.22	1.52
12	1.0	4.0	1 " "	18.2			1.15	
13	0.4	—	1 " "	34.0			0.42	
14	0.4	4.0	1 " "	27.0	20.8	0.9	0.62	0.94
15	0.4	4.0	1 " "	27.0			0.62	

* The figures in these columns are not the actual concentrations, but those to which they would correspond in blood analysis, namely, five times as much.

** At 4 hours.

The first four experiments of this table were designed to ascertain what intensity of color may be produced within short periods of time by glucose alone. For this purpose a creatinine standard was inconveniently dark, and the one used was a simple picric acid solution treated with the regular amount of alkali. Bearing in mind the very light tint of such a standard it will be seen that the glucose of a normal blood (Experiment 1) could yield very little color in an hour, and no more than a practically negligible quantity within 10 minutes. Elsewhere[4] we have shown that the color of the sodium picrate standard is about two and a half times less intense than that of a "1 mg." creatinine standard. It may therefore be calculated that on the

scale of the latter the glucose color at 10 minutes in Experiment 1 would be equivalent to $(20 - 18.3) \times \frac{2}{5} = 0.7$ mm., which corresponds to something like 0.05 mg. of creatinine. This estimate is confirmed by Experiments 7 to 10, in which the maximum effect, upon the 1 mg. standard, of a glucose concentration comparable to that of normal blood, is seen to be only 0.5 mm., corresponding to 0.04 mg. of creatinine.[13] A greater effect becomes of course noticeable in time. Within an hour it would correspond in a normal blood to about 0.14 mg. of creatinine (average of Experiments 7 to 9), in 24 hours to 1 to 2 mg. (Experiments 6 and 7).

Quantities of glucose greater than normal react with considerably greater rapidity. According to Experiments 11 to 15 a concentration equivalent to 0.4 gm. per 100 cc. of blood may in 10 minutes yield enough color to simulate 0.15 to 0.22 mg. of creatinine; the apparent increment may amount in 1 hour to 0.5 mg.; and after 24 hours the solution will be so dark as to be almost opaque. It is clear therefore that the glucose factor may assume considerable importance whenever there is a notable hyperglycemia, and it is quite possible that some of the creatinine values reported in cases of diabetes and of nephritis may on this account have been slightly exaggerated. But our results with normal proportions of sugar make it exceedingly doubtful that these could, within 10 minutes, produce any effect of consequence, or that the addition, as recommended by Gettler, of equivalent amounts of glucose to the standard is a prerequisite of a successful creatinine determination.

A point which is not directly important in the present connection, but which may be mentioned parenthetically, is that the glucose color is less permanent than the creatinine one, and that side by side with its gradual development there takes place, presumably as the result of oxidation, a gradual fading. The amount of glucose color present at any given period depends therefore on circumstances which are not always entirely controllable. This is illustrated in Experiments 6 and 7, where two different readings are reported for the 24 hour period. Of these

[13] The colorimeter readings of the table, like all which we may have occasion to report, are interpreted by reference to a standard curve constructed by and for the individual observer.

the lower one in each case was given by a portion of the mixture which had been kept in a tall narrow test-tube, the higher by one which had been exposed in an Erlenmeyer flask to a greater surface of air. This susceptibility to reoxidation (if that be the correct explanation) may account for the fact that although in actual determinations of blood or plasma creatinine the color is usually deeper at 24 hours than at 10 minutes, the extent of its increase is very variable and not infrequently it is actually lighter.

To return to the time curve, the indications of which are merely confirmed by the other experiments reported, it will be seen that the glucose effect, negligible as it may be in practice, does not leave the form of the curve altogether unaffected. Indeed, to judge from the glucose experiments, the method of time curves is rather more delicate than we had expected it to prove. The expression k, as calculated from the data for the first 10 minutes, is no longer a constant, being distinctly higher at first than it afterwards becomes. The glucose component causes the curve to begin its rise a little more steeply than the pure creatinine one; it abolishes the maximum at 9 minutes, although at that point the completion of the creatinine reaction is not entirely concealed; and it induces a subsequent gradual and prolonged rise, proceeding apparently at a constant rate so long as the observations were continued.

Although all four of the curves, which were combined into the one shown, exhibited these characters with greater or less distinctness, they did not coincide with one another so entirely as the pure creatinine ones. This slight variability is in accord with the additional results reported in Table II. It is not pronounced enough to influence materially any conclusion we have drawn from the composite curve.

Plasma.—The plasma curve shown in Chart I represents the average of eleven experiments on as many individuals. As it happens, the average creatinine content of these, assuming the correctness of the Folin method, was not far from 1 mg. (1.06 mg.) per 100 cc. This greatly facilitates direct comparison of our plasma curve with those for creatinine and for creatinine with glucose. It will be seen that it assumes a form intermediate between the two. Like the latter it rises rather steeply at

first, yielding in the first few minutes higher values for k than in the later ones. Like the latter also it exhibits after the 9 minute point a continued rise, during which indeed the resemblance increases to an almost absolute parallelism. But its deviation from the logarithmic formula with a constant k of 0.46, although unmistakable, is distinctly less marked than that of the curve with glucose. In fact, from the 3rd or 4th minute to the 9th the plasma curve becomes indistinguishable from a pure creatinine one. It is clear that the differences existing between plasma and pure creatinine solutions are more than accounted for by the glucose content of the former; and it is difficult to resist the conclusion that the determination of creatinine in a plasma filtrate is likely to be free of serious error. This conclusion, we shall see, is confirmed by the other tests which we have applied.

Whole Blood (Folin Procedure).—The eleven experiments which contributed to the construction of this curve (Chart I) were carried out on the same specimens that furnished the eleven plasmas of the preceding one. The time curves for whole blood and plasma are therefore directly comparable with one another. Since, moreover, the average apparent creatinine content of the group of whole bloods was 1.57 mg. per 100 cc., the time curve for the 1.5 mg. standard furnishes another convenient basis of comparison.

In accordance with data already reported by us in a preliminary communication,[1] and since then supplemented by many others not yet published, the curve for whole blood lies at a considerably higher level than that for plasma. It assumes, at the same time, a decidedly different form. In every respect it departs yet further from the monomolecular formula. It begins its rise even more steeply, shows an even greater variability of k, passes the 9 minute point with an even less conspicuous change of direction, and continues with an even more decided upward trend thereafter. The contrast between the two curves is perhaps most obvious to the eye in the interval from the 9th to the 15th minutes, in which the apparent creatinine content of the blood increases 0.13 mg., that of the plasma only 0.04.

Since at the 9th minute the creatinine reaction is complete,

the color developing subsequently, as well as that which distorts the earlier part of the curve, must be due to something else. This something else cannot be merely glucose, for blood contains no more glucose than plasma, and the curve for the latter, as well as that for creatinine with glucose, has already given us the measure of the glucose effect. The obvious deduction is that we are dealing here with some unknown substance, present in the corpuscles only, reacting slowly as compared with creatinine, but rapidly enough to produce an appreciable result within the time prescribed for the colorimetric comparison. This deduction may not be entirely sound; it is at least conceivable that the effect is a glucose effect after all, but one which has been catalytically accelerated by something of corpuscular origin. The point is immaterial. What matters at present is the result, and that is clearly such as to render the colorimetric determination of creatinine in whole blood subject to a decided error of overestimation.

The probable magnitude of this error can be roughly gauged from the form of the curve. Between the 9th and 10th minutes the unknown substance contributes color enough to counterfeit 0.03 mg. of creatinine. In the preceding 9 minutes there must therefore have developed the equivalent of at least 0.27 mg. This would reduce the content of actual creatinine from 1.54 (the apparent amount at 9 minutes) to 1.27. Even this estimate is still a maximum. The unknown reaction, there is every reason to suppose, proceeds with greatest rapidity during the first few minutes. Its time curve, displayed without complication by creatinine between the 9th minute and the 40th, will be found on analysis to be a logarithmic one (with a velocity constant of about 0.094) which, when projected backwards, as shown by the dotted line on the chart, cuts the zero abscissa as low as 1.15 mg. This, then, is probably the true value of the creatinine component of the combined curve. If it be accepted as such, the value found by the Folin procedure (1.57 mg., reading at 10 minutes) would be an overestimate of 36.5 per cent. The apparent excess of creatinine possessed by whole blood in comparison with plasma is by this conclusion very sensibly diminished.

Upon the quantitative details of the foregoing argument we

do not desire to lay more stress than the derivation of the curve would warrant. It is not pretended that the rather minute analysis attempted has the force of a mathematical demonstration. Our claim is merely that it proves the existence of a not entirely negligible disturbing factor, and enables us to form a probable estimate of its average effect.

The difference between the reactions with whole blood and with plasma was illustrated not only by the composite curves reproduced but also by every one of the eleven pairs of individual curves constructed. Especially obvious in each case was the lack of parallelism between the 9th and 15th minutes. This was not always so pronounced as in Chart I, but on the other hand it was often very much more so. It would appear therefore that the error of the whole blood determination may be in different specimens greater or less than the average estimate which we have reached. Seldom, if ever, can it be entirely absent.

This conclusion is fortified, as we shall see, by the results of the other tests employed; and it receives indirect support from the yet more striking issue of the experiments with the Myers' technique.

Whole Blood (Myers' Procedure).—Wilson and Plass[8] have recently stated that when in the determination of creatinine the blood is laked before being saturated with picric acid, the values obtained are usually higher than those yielded by the original procedure of Folin. This statement we have tested and abundantly confirmed; indeed we have not encountered any human blood to which it did not apply. In extreme cases the creatinine content of laked blood has even appeared to be more than double that of unlaked.[14] Upon this phenomenon we have endeavored to throw some light by constructing time curves of the reaction for this particular case also.

The curves obtained differed strikingly from those given by unlaked blood. They differed so much that if we had utilized all of our single curves in making the composite one, the latter would have passed the limits of Chart I within the first 10 minutes. We therefore made a selection, of two curves only, which

[14] We have received the impression that the blood of pregnant women is especially likely to show extreme differences between the results by the Folin technique and those by the other.

would yield a composite free from this inconvenience. The specimens from which these selected curves were derived had an average creatinine content by the Folin procedure (1.53 mg.) not far from the average for the whole eleven. The composite can therefore, in spite of the limited number of observations upon which it is based, be fairly enough set by the side of those already discussed. Specially selected though it is, its whole course cannot be displayed upon Chart I. It is therefore repeated and completed on Chart II, which starts from a higher base line than the other. It is a curve, as will be seen, which exhibits in most respects merely an exaggeration of the characteristics of that for unlaked blood. In both the coefficient k presents initial values which are higher than the constant of the creatinine reaction, to be followed by later ones which sink below it; but with laked blood each of these effects, and especially the latter, is so much more prominent that the curve retains hardly a suggestion of the logarithmic form. Its course suggests that the cause of the higher values given by laked blood is the relatively rapid development of a secondary reaction which, adding itself to the creatinine one, continues with but slightly abated velocity long after the latter must have come to an end. The curve therefore not only illustrates admirably the statement of Wilson and Plass, but settles the question which they left in doubt, whether the deeper color yielded by laked blood is due to the "liberation of creatinine which is otherwise occluded in the voluminous precipitate, or merely the liberation of color-producing material not creatinine." Whatever the responsible substance may be, it is evidently not creatinine. One cannot help suspecting that it is the same substance which we have already encountered in unlaked blood, and that the destruction of the corpuscles has simply set it free in greater amount. This idea is at least not contradicted by the similar behavior of the coefficient k in the two cases. It is quite probable that even when picric acid solution is added directly to blood, some laking takes place; and perhaps if we could prevent it altogether the determination in whole blood might be made as accurate as that in plasma.

We have on a former occasion[4] commended the Myers' technique as obviating, at one point at least of the determination,

the danger of introducing a picric acid solution which has deteriorated through exposure to light; but the facts now revealed leave no doubt in our mind that the Folin procedure is to be preferred, and that results obtained by the other are always somewhat, and often very much, too high. They will suffer from this defect even when the strictest attention is paid to the time limit for the color comparison; while, at such a rate does the development of the extra color continue, that the accident of delay as a cause of error assumes greater importance than ever.

Autoclaved Plasma and Whole Blood.—To the color reaction in autoclaved blood and plasma, upon which depends the Folin determination of creatine in these fluids, we have devoted only a few experiments under the present head. This was partly because, as will be shown later, the question of the accuracy of this determination appeared to be settled decisively enough in another way, and partly because, before we reached this point in our inquiry, it had been made the subject of published investigations by others.[8,9] Nevertheless it seemed worth while to make one or two time curves in these cases also, if it were only to complete the series, or to control the conclusions reached by other methods. Incidentally the attempt was rewarded by the development of some rather interesting points.

We present therefore in Chart II composite curves for "total" creatinine, derived respectively from two whole bloods and two plasmas. A standard of comparison for each was provided by calculating on the basis of previous observations the course which the reaction would have followed if it had been a pure creatinine one. The admissibility of such a procedure has already been demonstrated. The calculated curves are indicated by interrupted lines. It should be noted, in comparing Charts I and II, that in the latter the base line is not zero, but 1.0 mg.

The curves for unheated blood and plasma have revealed the existence of a disturbing factor peculiar to the former. This factor is probably present in autoclaved blood also. But as the determination here is always carried out in a greater dilution of the picric acid filtrate, its influence might be expected to be relatively much less prominent. Moreover, as this factor is absent from plasma, one would be prepared to find the curve for total plasma creatinine distorted only by the diminished con-

centration of sugar still present. Neither of these expectations appears at first sight to be fulfilled. On the contrary both curves exhibit a rather greater departure from the normal than any we have yet examined. There is therefore here some new element of error in operation. On a casual inspection of the curves its effect seems to be of the same kind as that already recognized. A closer scrutiny reveals a distinction. The curves now under consideration appear to spring not from the zero point of the creatinine scale, but from a higher one; from somewhere near 1.12 in the case of whole blood, and 1.03 in that of plasma. This would mean that the extra color complicating the creatinine reaction is in the present case one which develops instantaneously upon the addition of the alkali. This idea is supported by the fact (commented upon already by Wilson and Plass[8]) that the growth in depth of the color is in autoclaved blood filtrates visibly much more rapid than in untreated ones. It is supported also by dissection of the curves with the aid of the coefficient k. This assumes at first the highest values yet observed, and remains, even in its subsequent decrease, above the level of the creatinine constant. If, though, at each point of the curve there be deducted from the observed creatinine value a constant quantity of 1.12 mg. for blood and 1.03 mg. for plasma, and if corrected values for k be calculated upon the fresh basis thus arrived at, it will be found that these form in either case a series which differs less actually from the standard fixed by pure creatinine than do the figures for unheated blood or even plasma. This is made evident in Table III, where the corrected coefficients (designated, to prevent confusion, k') are exhibited alongside of the original ones, taken from Table I, for creatinine and unheated plasma respectively. It may be remarked that the corrections introduced affect, in the formula for k', the values of a and x, but not of $a - x$.

While once more disclaiming for such calculations any exaggerated importance in a quantitative aspect, we think that they render exceedingly plausible our suggested interpretation of the curves, that, namely, they are simple creatinine ones superimposed upon an initial color instantaneously produced by something else. If this view of the matter be indeed correct, we can form an approximate estimate of the absolute and relative magni-

TABLE III.

Corrected Coefficients (k') for "Total" Creatinine Curves.

$$k' \text{ (blood)} = \frac{1}{t} \log_e \frac{1.70}{a - x}$$

$$k' \text{ (plasma)} = \frac{1}{t} \log_e \frac{1.25}{a - x}$$

t	k		k'	
	Creatinine (average).	Plasma.	Autoclaved blood.	Autoclaved plasma.
min.				
1	0.47	0.70	0.56	0.69
2	0.47	0.55	0.48	0.51
3	0.46	0.50	0.44	0.51
4	0.45	0.47	0.44	0.46
5	0.44	0.46	0.45	0.44
6	0.45	0.46	0.47	0.44
7	0.47	0.44	0.50	0.46
8	0.52	0.49	0.55	0.47
9	0.53	0.52		0.54
10				

tude of the error in creatine determinations. The whole blood filtrates, after autoclaving, had been diluted for the colorimetric comparison in the proportion of 3 : 10; the blood therefore contained apparently an average of 9.40 mg. of "total" creatinine; deducting from this $\frac{1}{3}^{0}$ of the assumed correction (1.12) = 3.73 mg. we reach a true creatinine-creatine content of 5.67 mg. As the preformed creatinine of the same bloods was found to be on the average 1.59 mg., the creatinine from creatine was 7.81 mg. according to the original observation, and 4.08 according to the corrected one. The error in the estimation of creatine amounts therefore to 91 per cent. For the plasmas the dilution was 4 : 5, the assumed correction 1.03 mg., and the preformed creatinine 1.08. From these data may be similarly calculated a true "total" creatinine content of 1.56 mg., a true creatine of 0.48 mg., and an observational error of 1.29 mg., or 270 per cent. Roughly, it may be concluded, the true creatine content of whole blood is likely to be about half, that of plasma about one-fourth, of the amount indicated by the Folin method. Whatever value these estimates may possess, the existence of serious errors in both cases would appear to be thoroughly established.

Though the initial color of our hypothesis may be the main source of these errors, it can hardly be the only one. The continued increase of color which follows the main reaction indicates that the factors operating in determinations of preformed creatinine are almost certainly also still in action. In the case of whole blood they have been so diluted as to become practically negligible, for the curve after the 10th minute becomes an all but horizontal line. We have over and over again observed that the delayed color change is smaller with whole blood creatine determinations than with any other. With plasma it seems to have become, as a result of the autoclave treatment, if anything more prominent than before; but the observations on this point were too limited in number to give much significance to this apparent inconsistency.

The Effect of Dilution upon the Determination.

The amount of preformed creatinine in plasma, or even in whole blood, is already so small that the effect of dilution upon its determination could not be conveniently tested in specimens containing only the normal proportion. In estimating the magnitude of this effect it was necessary, therefore, to rely largely upon experiments with the blood of nephritics. The fact that such blood contained without doubt an abnormal accumulation of many other substances than creatinine would, it may be supposed, render the tests only the more severe.

Apart from this detail the plan of the experiments hardly calls for description. A determination was first carried out on the usual fivefold, or in the case of total creatinine ten- or fifteenfold dilution of the blood. Thereafter the blood filtrate was further diluted with selected proportions of saturated picric acid, and other determinations were made. To avoid complicating the result with the possible influence of variables other than the concentration, the standard employed with any one set of experiments was always the same, and the dilution was never carried beyond the limits within which that standard could be suitably applied.

The results obtained in many such experiments are illustrated by the group reported in Table IV. This constitutes a complete series upon a single specimen of blood, that of a nephritic, K,

who furnished material also for some of the experiments reported in the next section. The selection of this series for reproduction is made particularly appropriate by the fact that it includes for each application of the method instances of the maximum as well as the minimum error encountered. Table IV is therefore justly representative of the whole number of experiments performed.

We have come to the conclusion, as the result of considerable experience, that the inherent error of the colorimetric procedure,

TABLE IV.

Influence of Dilution upon the Creatinine Determination.

	Dilution factor.*	Creatinine per 100 cc.**		Error.	
		Found.	Expected.		
Preformed creatinine.					
		mg.	*mg.*	*mg.*	*per cent*
Whole blood (Folin).	1	3.76	—	—	—
	1.5	2.62	2.51	+0.11	+4.4
	2	1.86	1.88	−0.02	−1.1
Whole blood (Myers).	1	5.18	—	—	—
	1.8	2.68	2.88	−0.20	−7.0
	2	2.20	2.59	−0.39	−15.1
	2.5	1.94	2.07	−0.13	−6.3
Plasma.	1	3.85	—	—	—
	1.5	2.55	2.57	−0.02	−0.8
	2	1.85	1.92	−0.07	−3.6
Total creatinine.					
Whole blood.	2	4.40	—	—	—
	3	2.94	2.93	+0.01	+0.3
	4	2.23	2.20	+0.03	+1.4
	5	1.72	1.76	−0.04	−2.3
Plasma.	2	3.08	—	—	—
	3	2.02	2.05	−0.03	−1.5
	4	1.47	1.54	−0.07	−4.5

* 1 signifies the usual fivefold dilution of the blood.

** If multiplied by the dilution factor, gives the amount per 100 cc. of the blood concerned.

when applied to very dilute solutions of pure creatinine, and when using standard curves to interpret the readings, may be as much as, but should not be greater than 5 per cent. If this estimate of the permissible error be accepted, it will be seen that variations in the extent to which the blood is diluted affect the determination of creatinine in one case only, that, namely, in which the corpuscles have been laked before the addition of picric acid. This emphasizes the liability to error of that particular variation of the technique. Otherwise the application of the dilution test reveals no distinction between the several cases under consideration, and fails therefore to provide any assistance in solving the main problem. The experiments performed were nevertheless not entirely fruitless, for they provide a check upon those which are next to be discussed, and add to the significance of their very different outcome.

The Influence of Different Standards upon the Determination.

In order to ascertain how far any or all of the determinations met the third criterion proposed (that a change of standard should not affect the result), we have adopted one or other of the following plans.

1. When the amount of blood filtrate available was sufficient (experiments with subjects K, Cr, S, A, and B in Table V) the comparison of results with different standards was effected by making two separate experiments; in each due care was taken to use the same amount of alkali and to make the reading at exactly 10 minutes after its addition; but while in one the standard of comparison used was "1 mg." of creatinine, in the other it was "2.5." In most instances the two experiments were performed at the same dilution, one so chosen that the actual creatinine content lay conveniently between the two standards. In some (as in Experiments 14 to 17 among those reported) the dilution was varied as well as the standard; but we have already seen that dilution, *per se*, is without significant effect.

2. If too little blood had been secured to make the above procedure possible (subjects Ca and H of Table V), the reading was first made with a 1 mg. standard; this was then as quickly as possible replaced by a 2.5 one, and a second reading taken; finally

the first standard was returned to the colorimeter, and a last reading secured. The average of the results by the first and third observations (never, of course, very far apart) was compared with that by the second. Due attention was always paid to the obvious condition, that the dilution at which the determination was made must be one appropriate for both standards.

The manner in which the various determinations, with which we are concerned, responded to one or the other of these tests is shown by the data of Table V. These are selected from ex-

TABLE V.

Effect upon the Determination of Using Different Standards.

	Subject.	Experiment No.	Creatinine per 100 cc. according to:		Error of second determination as referred to first.		Average error of group.
			1 mg. standard.	2.5 mg. standard.			
Preformed creatinine.							
			mg.	*mg.*	*mg.*	*per cent*	*per cent*
Whole blood (Folin).	Ca	1	1.79	1.95	+0.16	+8.9	
	H	2	1.72	1.91	+0.19	+11.0	
	H	3	2.02	2.24	+0.22	+10.9	
	H	4	1.80	1.96	+0.16	+8.9	
	K	5	3.60	3.72	+0.12	+3.3	+8.6
Whole blood (Myers).	K	6	4.45	4.85	+0.40	+9.0	+9.0
Plasma.	H	7	1.23	1.29	+0.06	+4.9	
	H	8	1.37	1.42	+0.05	+3.6	
	H	9	1.37	1.37	0	0	
	H	10	1.48	1.48	0	0	
	K	11	3.72	3.70	−0.02	−0.5	+1.6
Total creatinine.							
Whole blood.	K	12	8.20	8.92	+0.72	+8.8	
	K	13	7.87	8.50	+0.63	+8.0	
	Cr	14	6.19	7.11	+0.92	+14.9	
	S	15	5.57	7.00	+1.43	+25.6	
	A	16	6.60	7.65	+1.05	+15.9	
	B	17	6.35	7.25	+0.90	+14.2	+14.6
Plasma.	K	18	5.19	5.94	+0.75	+14.5	
	K	19	5.24	5.72	+0.48	+9.2	
	Ca	20	2.96	3.40	+0.44	+14.9	
	Ca	21	2.86	3.31	+0.45	+15.7	+13.6

periments of which the total number was sufficient to give us confidence in the truly characteristic nature of the results within each group. As with Table IV, the selection is so made as to exhibit the extremes of error manifested by each of the special cases.

The results are so unequivocal that very little discussion of them is required.

With the preformed creatinine of the plasma it is clearly a matter of indifference what standard is employed. The absolutely exact agreement of some pairs of figures is of course a coincidence only. The point to be emphasized is that in none of them does the divergence exceed 5 per cent. The errors, in short, are no greater than they might be with pure creatinine solutions.

No such satisfactory accord is encountered in any of the other groups of analyses. Thus, with whole blood the outcome of the preformed creatinine determination shows an unmistakable dependence upon the choice of standard. If the 1 mg. one be assumed to give the correct result, the other yields a figure which on the average is nearly 9 per cent too high. This is an error rather too considerable to be accidental. Perhaps if it were the only evidence impugning the accuracy of the determination, one would not be prepared to consider it of decisive import; but taken in conjunction with that afforded by the time curves, it acquires indubitable significance. The fact that in one experiment (No. 5) the difference between the two results fell within the permissible range is only in appearance an inconsistency. The subject of that experiment, K, who was also the subject of all the experiments of Table IV, was one whose blood contained an abnormally high concentration of creatinine. To this the unknown agent of error bore therefore in all probability a smaller ratio than it does in normal blood, and it would be natural that it should exert a proportionally smaller influence in the analysis. Be this as it may, even the result with K does little to modify the general effect of contrast received on comparing the data for whole blood with those for plasma. The latter invariably met with success the conditions of the test; in every instance but one the former failed to.

Failure yet more decided is the outcome of every experiment

with "total" creatinine, whether in plasma or in blood. No distinction between the two cases is here apparent. Neither in one nor the other do the results with different standards ever come within reasonable range of one another. There is no escaping the conclusion that one at least of each pair of determinations must be quite considerably in error. From the figures reproduced it would appear that in the determination of creatine by the method of Folin a variation of about 1 mg. per 100 cc. with whole blood, and 0.5 mg. with plasma may depend entirely upon the choice of standard made. This is independent of the still greater intrinsic error, the existence of which was made probable by examination of the time curves.

The technique involving the laking of blood prior to saturation with picric acid had revealed such decided shortcomings in the tests already applied that we made but a single experiment with it under the present head. This was one of a series, consisting of Experiments 5, 6, 11, 13, and 19, which is perhaps of special interest in that it includes one example of each determination, all carried out upon a single specimen of blood. The blood was taken from the patient K, to whom we have already referred. The errors throughout the series are less than the averages for normal blood, a fact probably to be explained by the consideration already set forth in connection with Experiment 5; but they exhibit much the same ratios among themselves, and illustrate quite as well as do the final averages the relative position of the different cases. Among them the error of the experiment with laked blood is the highest of all. The issue serves therefore only to strengthen the unfavorable impression already formed of the Myers' technique.

The cases in which variation of the standard has been found to affect the result of the analysis are precisely those in which examination of the time curves has already revealed appreciable sources of error. The two sets of results are therefore mutually confirmatory. Their harmony extends even to the point of indicating for the several determinations the same order in relation to the magnitude of their probable error. It would appear, accordingly, very likely that the factor which makes it hard to obtain consistent results with different standards is the same as that which causes distortion of the time curve, the development,

namely, of extra color of unknown origin. To account more fully for the difficulty in relation to the standards, it would be sufficient to suppose that this extra color, whatever its source, is always of somewhat different quality from the creatinine one. This is more than a mere supposition. As a matter of fact the existence of abnormal shades of color is frequently obvious enough to the eye during the colorimetric comparison. Wilson and Plass[8] have already drawn attention to the difficulty encountered in matching the blood color with the standard in total creatinine determinations in whole blood; in our experience the difficulty is usually (and here we have to dissent from the opinion of Wilson and Plass) just as great with plasma. A similar difficulty, not so pronounced, is sometimes encountered with the preformed creatinine of whole blood. With the preformed creatinine of plasma matching is always practically perfect. To the necessarily rather vague impressions conveyed by such observations our experiments have added precision and something at least of quantitative meaning.

Since a disagreement between results with different standards is due to something added to the creatinine, it is evident that the lower of the two estimates is always the closer to the truth. The lower result, it will be noticed, is always the one obtained with the 1 mg. standard. It follows that, while the Folin method for creatine gives results that are in any case too high, the error will be minimized by diluting the blood or plasma always so far at least as to bring the color comparison within the range of the 1 mg. standard. It is an additional argument for this recommendation that (to the eyes at any rate of both the present writers) any existing difference of color quality is less obtrusive, and the process of matching is accordingly easier, with the lower standard than with the higher.[15]

[15] It should be stated that it is our invariable practice to set our standard, whether 1 or 2.5 mg., at 20 mm. When the stronger one is set at only 10 mm., matching of slightly dissimilar shades is apparently easier, and presumably the two standards would with such an arrangement give results that are closer together. It is perhaps not inopportune to point out further that, when the colors are not exactly alike, greater play than usual is given to the influence of the personal equation. These remarks have an obvious bearing upon the possibility of reproducing in detail our results with different standards; but they cannot affect the significance of a whole series of results by one observer using invariably the same technique.

Other Considerations Affecting the Accuracy of the Determinations.

The evidence so far considered bears solely upon the colorimetric aspects of the determination of creatinine in blood. Before it can be applied to the formation of a final judgment upon the points at issue, it is necessary to refer to certain possibilities of error in the preparatory manipulations which precede the actual colorimetry. There are two of these which have seemed to us to deserve some attention.

1. When, as the first step of the analysis, the blood is diluted, by the addition of saturated picric acid, to a volume five times greater than the original, that volume remains partly occupied by the bulky precipitate of proteins. The volume of fluid which now holds the creatinine in solution, and in which its concentration is ultimately determined, is therefore not five times the original one. The assumption that it is so will involve a not inconsiderable error of overestimation.[16] The extent of this error we have attempted to ascertain by determining, with the simplified hematocrit of Epstein,[17] what proportion of the total volume, after final saturation with picric acid, is occupied by the uniformly suspended precipitate. When the latter had contracted to a constant volume (which it did only after long continued centrifugation) it was found to account for, on the average, with whole blood 12 and with plasma 8 per cent of the entire volume of the mixture. Experiments with different individuals showed only inconsiderable variations from these averages. To compensate the error caused by neglecting the precipitate, it would be necessary, therefore, to deduct the above mentioned percentages from the creatinine values as ordinarily reported. The more voluminous character of the precipitate with whole blood, involving a consequently greater error in its case, is evident even in the course of the ordinary centrifugation, which forms part of the regular routine of the determination.

2. An error operating in the opposite direction is caused by the slight dilution of the blood that results from the addition of potassium oxalate solution. If the directions of Folin, to

[16] Unless indeed some creatinine is adsorbed by the protein precipitate, a point on which we possess no information whatever.

[17] Epstein, A. A., *J. Lab. and Clin. Med.*, 1916, i, 610.

which we have in this respect strictly adhered, be followed, this dilution will in the case of whole blood amount to ten drops (= 0.5 – 0.6 cc.) in 30 cc., which is about 2 per cent. It is more difficult to estimate the exact effect on the plasma. It is of course the plasma alone which directly gains in volume when blood is diluted. The corpuscles, in consequence of the osmotic effect of the added oxalate, actually shrink. The plasma is therefore diluted not only by the water added from without, but also by fluid passing into it from the corpuscles. In order to gain some idea of the total addition to the plasma which may thus be produced we have compared in the same blood the plasma volume after (1) treatment with hirudin alone, with that after (2) the addition, as in the usual technique, of one-fiftieth volume of 20 per cent potassium oxalate solution. As the average of several such comparisons we found that a plasma volume of 57 per cent in hirudinized blood increases to 62.5 per cent in the oxalated specimen. This implies the addition to the original plasma of roughly 10 per cent of its volume. Unfortunately one cannot at once argue that the creatinine concentration of the plasma is correspondingly diminished. For it is possible, indeed likely, that the corpuscles are permeable to creatinine, and that the passage of that substance through the corpuscular envelope will ultimately compensate any effect of dilution. It is impossible to say how long it may take to bring about such a redistribution of creatinine. One can therefore hardly venture to estimate numerically the influence of the oxalate upon creatinine concentration in the plasma. The most one can say is, that it must at least partially compensate the error due to the protein precipitate, and that it may possibly neutralize it altogether. Under the least favorable assumption, that of complete and instantaneous redistribution of the creatinine, it will reduce the necessary correction from 8 per cent to 6.

With whole blood it is possible to estimate more precisely the net effect of these opposing influences. To allow for both, it will be necessary to deduct 12 – 2 = 10 per cent from the observed value. Conversely, the latter, with reference to the truth, is an overestimate of 11 per cent.

The influence of oxalate cannot be dismissed without reference to its so called "bleaching" effect upon the creatinine color.

That excessive amounts of oxalate may be a source of danger in this respect was pointed out by Folin in his original description of the method; but according to Gettler[7] the effect is noticeable even when the quantity employed is less than double the prescribed proportion. Although we have tested the influence of oxalate upon the color in three different ways, we are unable to confirm this statement.

Thus, in one experiment a saturated picric acid solution containing 0.5 mg. of creatinine per 100 cc. (a "2.5 mg." standard) was divided into two parts. To one was added 20 per cent potassium oxalate in the proportion of ten drops per 100 cc., which is rather more than the amount which a blood filtrate would regularly contain; to the other was added an equal proportion of water. Both were treated with the proper quantity of alkali, and immediately compared with one another in the colorimeter. Neither at the first nor within the course of the next 3 hours was the slightest difference between them to be detected. The oxalate had no effect whatever.

In another experiment we measured from a specimen of defibrinated ox blood four 10 cc. portions, to which we added respectively 0, 3, 6, and 12 drops of 20 per cent oxalate solution. A creatinine determination was then carried out on each. The results were practically identical, for they appeared to contain respectively 1.60, 1.65, 1.65, and 1.60 mg. per 100 cc. According to this experiment as much as four times the usual amount of oxalate may be employed with very little, if any, effect upon the result.

A third test consisted in directing human blood from an arm vein partly into a vessel containing a little 20 per cent oxalate solution, partly into one supplied with a flake or two of hirudin. Each part was divided into a smaller and a larger fraction, and from the latter the plasma was separated in the centrifuge. Each specimen of whole blood and of plasma thus obtained was finally analyzed for creatinine by the Myers' procedure. The whole experiment was repeated with a second subject. In each determination the standard was a "1 mg." one set at 20 mm. The colorimeter readings are shown in Table VI. It will be seen that the presence or absence of oxalate has little effect upon any one of the results. Such differences as appear are not greater than can be readily account-

ed for by the merely diluent action of the oxalate solution added. We are therefore of the opinion that in all ordinary circumstances this effect of dilution is the only one that need be taken into account.

TABLE VI.

Effect of Oxalate upon the Creatinine Color in Blood and Plasma.

	1 mg. standard.	Hirudinized.	Oxalated.
	mm.	*mm.*	*mm.*
Whole blood A	20	14.0	14.7
Plasma A	20	18.1	18.5
Whole blood B	20	13.7	14.7
Plasma B	20	17.4	18.2

DISCUSSION AND CONCLUSIONS.

It has been shown that of the four or, including the Myers' modification, five applications of the Folin method for blood creatinine only one meets successfully each of the tests which we proposed. While it is admitted that these tests are incapable by themselves of furnishing any absolute demonstration of accuracy, one may nevertheless, in accordance with our main argument, infer that the colorimetric part at least of that particular application is in all probability free from serious error. The case thus specially distinguished from the others is that of the determination of *preformed creatinine* in *plasma*. Since for the same case it has been further shown, that the merely manipulative imperfections of the technique must all but neutralize each other's influence, the substantial accuracy of results upon the creatinine content of plasma would seem to be established. The net effect of all the relatively unimportant sources of error detected (glucose, protein precipitate, and dilution by oxalate) can hardly amount to 10 per cent, and may be very much less.

In all the other circumstances in which the colorimetric determination of creatinine is applied to blood analysis, it fails more or less completely to satisfy two out of the three requirements laid down. Failure in one would be sufficient to convict the method of inaccuracy. As to the relative extent to which its usefulness is affected in the different cases, both of the available criteria yield, it has been pointed out, identical implications.

In the case of the *preformed creatinine* of *whole blood* the error is considerable, yet perhaps not, in view of all the circumstances, immoderately large. The time curve of the reaction indicates, we have seen, that colorimetry exaggerates the creatinine content of the blood by, on the average, 36.5 per cent. Moreover, the minor sources of error inherent in the operations preliminary to the actual determination cannot with whole blood be assumed to cancel one another. Rather they involve a further error in the positive direction amounting to something like 11 per cent. The cumulative effect of these errors will be that the preformed creatinine of whole blood will be estimated at 150 per cent of its true value.[18] Conversely, to reach the probable true value from the observed, one must diminish the latter on the average by one-third. It may be noted that if this conclusion be applied to the average creatinine content of the eleven bloods from which we constructed our time curve, it will be reduced from 1.57 to 1.05, which is identical with the average creatinine content of the plasmas.

The opinion that the Folin method exaggerates the creatinine content of whole blood has already been expressed, or at least implied, by Wilson and Plass.[8] In support of it they offer very little positive evidence. As to the extent of the exaggeration they content themselves with the unsupported statement or assumption that the differences between the observed creatinine contents of whole blood and plasma are "sufficiently small to be ascribed to the anomalous color development when whole blood is used." Our observations place these, as we believe, perfectly correct conclusions upon a decidedly firmer basis, and furnish real evidence on which to discuss the question of the distribution of creatinine in the blood. To that question we propose to return in a later communication.

Our estimate of the probable error of whole blood creatinine determinations applies only to the original technique of Folin.

[18] Of course it should not be forgotten that our manner of using the Folin method differs in one respect from the original; namely, in the use of interpretative curves. With most normal bloods this has the effect of giving a higher result than the usual calculation according to the rule of inverse proportionality. The error of the absolutely unmodified Folin procedure will therefore generally be less than the estimate applicable to our own results.

When the blood is laked, all our observations agree in indicating that the error is considerably greater. Even the dilution test, successfully passed by all other cases, fails in this particular instance. The average error which results from laking cannot fairly be estimated from the limited number of data we possess; but the error of individual cases has been found to exceed 100 per cent.

For the accurate determination of *creatine* in *whole blood* the method of Folin would appear, on the evidence we present, to be quite inadequate. The experiments bearing upon this point are so consistent and so definite in their significance, that they may be regarded, in spite of their limited number, as thoroughly convincing. Calculations based upon the time curve of the reaction in autoclaved blood suggest that the true creatine content of whole blood may be only one-half of that indicated by the method. The absolute magnitude of such an error would amount to several mg. per 100 cc.

By devising new methods presumably free from the objections to which Folin's is liable, Wilson and Plass,[8] as well as Greenwald,[9,19] have already reached upon the question of blood creatine the same conclusion. Our results are to be regarded therefore mainly as a confirmation, from a different angle, of theirs. The new methods agree with each other, and with our own deductions, in indicating for the old one an error of the order of 100 per cent.

Plasma, as regards the determination of its *creatine*, would appear on the evidence of our tests to be on no better footing than whole blood. The probable error, as we have calculated it, is, absolutely considered, smaller than in the latter, but on account of the low creatine content of plasma relatively greater. It is indicated that the results of the Folin method in this application may be nearly four times too high. On this point our conclusion is in conflict with that of Wilson and Plass,[8] who say in reference to it that "the agreement of the results" (according to Folin) "with those of the acetic acid procedure . . . . . lends support to the accuracy of the method." It may, however,

[19] We are indebted to a private communication for the information that eight human bloods, of which the creatine content according to the method of Folin varied from 2.5 to 8.5 (average 5.3) mg. per 100 cc., contained according to that of Greenwald only 2.3 to 4.1 (average 3.3).

be pointed out that the agreement of which they speak does not appear in their published results to be a very close one. In one case (mixed specimen) the Folin method gives eight times as much as the 0.1 mg. given by the other. In two more (W, H) where the acetic acid procedure shows no creatine at all, the Folin indicates 0.4 to 0.6 mg. These are magnitudes which are absolutely small enough, but relatively to those with which Wilson and Plass are dealing by no means inconsiderable. In only four out of the nine human plasmas which they examined by both methods do the results actually agree.

It remains to be said, in conclusion, that while the verdict just passed upon certain applications of the Folin method for creatinine to blood analysis is, from the point of view of the strictest accuracy, far from favorable, it is by no means to be regarded as wholly condemnatory. Especially is this the case with the determination of preformed creatinine in whole blood. A 50 per cent error might here, according to the circumstances, have a very great significance or none at all. In a problem like that of creatinine distribution it becomes of crucial importance. For most of the purposes to which blood creatinine estimations have been applied it is of no consequence whatever. To the clinician it matters little whether a patient's blood contain 1.5 mg. of creatinine per 100 cc. or only 1.0; he is concerned to discover variations of much greater magnitude. The clinical usefulness of the method is the less likely to be affected, because the relative importance of the error probably, as indicated by our own observations on the patient K, diminishes as the creatinine concentration increases. Even with the technique of Myers, which proved to be quite useless for certain purposes we have had in view, it is possible, as its originator has abundantly proved, to obtain results of the greatest interest and importance. Only when it is a question of attaining the utmost possible accuracy will it become necessary or advisable to make the determination upon the plasma in preference to the blood.

In this aspect of the matter even the method for creatine, which in comparison with later improvements has the merit at least of simplicity, may possess a certain restricted usefulness. It is probable enough that its results vary in a general way with the true creatine content of the blood. The relative constancy

of the results obtained with normal subjects, and the generally observed increase in pathological conditions associated with retention of other blood constituents, would lend support to such an assumption. A series of comparative analyses in which the Folin method for creatine has been employed may therefore easily reveal real fluctuations in the concentration of that substance. Only, in order that this should be possible, or that different series should be in any sense comparable, it will be essential, as our experiments demonstrate, that the same standard be employed in every single determination.

SUMMARY.

Time curves are presented showing the rate at which the color utilized as the basis of a creatinine determination develops, not only in pure aqueous solutions, but also in the various circumstances in which it is applied to the analysis of blood. By comparison of these curves it is shown to be probable that:

1. The only substance in plasma capable of simulating the reaction for creatinine is glucose, and its influence upon the determination is too small to have much practical importance.

2. Whole blood contains in addition an unknown substance which, although reacting more slowly than creatinine, contributes in the Folin technique an appreciable fraction of the total color developing within 10 minutes.

3. The amount of this substance passing into the protein-free filtrate is greatly increased on laking the blood.

4. Autoclaved blood or plasma filtrates contain a substance reacting instantaneously upon the addition of alkali in such a manner as to simulate relatively substantial quantities of creatinine.

These deductions are corroborated by the way in which a change of standard affects the outcome of the several determinations studied.

After weighing the probable effect of certain manipulative imperfections of the method, it is finally concluded that:

1. The Folin method determines the preformed creatinine of plasma with a satisfactory approximation to accuracy.

2. The results of the same method upon the preformed creatinine of whole blood are on the average about 50 per cent higher than the truth.[18]

3. The technique of Myers leads to a still greater exaggeration of the preformed creatinine of blood.

4. The Folin method for creatine, whether applied to whole blood or to plasma, gives decidedly erroneous results, liable to be in the former case about twice, and in the latter about four times, as high as the amount actually present.

A MICRO METHOD FOR THE DETERMINATION OF CALCIUM AND MAGNESIUM IN BLOOD SERUM.

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The methods described below[1] were devised especially for the study of the composition of the blood of infants suffering from rickets and tetany, and are not recommended as substitutes for the usual gravimetric procedures where a sufficient amount of material is available. By these methods it is possible to determine calcium and magnesium in 2 cc. samples of serum with a maximum error of less than 5 per cent, a degree of accuracy entirely sufficient for the purposes for which the methods were devised.

Principle of the Methods.

The methods depend upon the fact that solutions of ferric thiocyanate are decolorized by oxalates and by phosphates. Calcium is precipitated as the oxalate and magnesium as the ammonium magnesium phosphate; the precipitates are dissolved in acid and added to solutions of ferric thiocyanate, the degree of decolorization resulting being determined by comparison in small Nessler tubes.

Technique of the Calcium Method.

2 cc. of clear serum are measured into a 50 cc. conical beaker, 10 cc. of concentrated nitric acid are added, and the beaker is heated on an electric stove just below the boiling point for 2 or 3 hours. The heat is then increased and the acid evaporated down to about 0.5 cc. Sputtering must be guarded against and the contents must not go completely to dryness.[2] The sides of

[1] A preliminary description of these methods appeared in the Proceedings of the American Society of Biological Chemists, *J. Biol. Chem.*, 1916, xxiv, p. xviii.

[2] If charring occurs, more acid must be added and evaporation repeated.

the beaker are washed down with 2 or 3 cc. of water and a drop of phenolsulfonephthalein is added as an indicator. Dilute ammonia is added drop by drop until alkaline.[3] The beaker is then replaced on the stove and heated until the excess of ammonia is removed, as shown by a change in color of the indicator and the fact that only a faint odor of ammonia remains. Unless a very great excess of ammonia has been added, 2 or 3 minutes at medium heat is usually sufficient. While still hot 1 cc. of a solution of 1.25 per cent oxalic acid in 0.25 N hydrochloric acid is added drop by drop and with stirring. The beaker is removed from the stove and when cool 0.5 cc. of sodium acetate solution (20 per cent)[4] is added slowly and with stirring. The solution is allowed to stand over night. This method of precipitation, which is essentially that advised by McCrudden,[5] results in a granular precipitate of calcium oxalate, that can be readily filtered without loss.

Filtration is carried out on a 10 cc. Gooch crucible, the mat being especially prepared as follows: A small disc of filter paper is first placed in the bottom of the crucible, asbestos soup is poured on to make a fairly thick mat, another disc of filter paper is laid on and then a little more asbestos, and finally a suspension of purified barium sulfate. This latter serves to make evident any leaks in the crucible and also to close the pores.

The calcium oxalate precipitate is washed into the crucible and beaker and crucible are washed eight times, each time with approximately 5 cc. of 1 per cent ammonia (one part concentrated ammonia in 100 of water), then once with 95 per cent alcohol, containing just enough ammonia to be alkaline, and finally once with ether.[6] The suction is best diminished when the ether is poured on the crucible so as to prevent excessively rapid filtration. After all the ether has passed through the filter, the suction is increased and after 5 or 10 minutes the mat is quite dry.

[3] The indicator changes from an eosin pink to yellow and then to reddish purple, the latter color being the alkaline end-point.

[4] This refers to the anhydrous salt. If crystalline sodium acetate is used the solution should be made up to a strength of 35 per cent.

[5] McCrudden, F. H., *J. Biol. Chem.*, 1909–10, vii, 83, 201; 1911–12, x, 187.

[6] Ether which contains acid should be redistilled over sodium hydroxide before using.

The crucible is returned to the beaker and 10 cc. of 0.2 N hydrochloric acid are run into the crucible and allowed to percolate through. The beaker is covered by a piece of rubber dam held in place by a rubber band or put in a desiccator over water, in order to prevent evaporation. After standing several hours or over night the asbestos is thoroughly stirred up in the acid and the whole suspension transferred to a tube and centrifuged. An aliquot portion of the clear supernatant fluid (usually 6 cc.) is pipetted off and used for the colorimetric determination.

Colorimetric Comparison.

Small Nessler tubes approximately 120 mm. long and 10 mm. internal diameter are used. They are graduated at 10 cc., and the bores of the tubes should be such that the graduation on each tube should be within 2 mm. of the graduation on any other tube in the set. Round-bottomed tubes may be used, but those with flat bottoms are preferable. Aliquot portions of the oxalate solution are measured into the tubes. Portions of a standard calcium oxalate solution[7] are measured into other tubes. Then to each tube are added 2 cc. of ferric thiocyanate[8] solution accurately measured with an Ostwald pipette. Each tube is filled to the mark with 0.2 N hydrochloric acid, and the contents are mixed by inverting several times, the ends of the tubes being closed with a clean rubber stopper.

Color comparisons are made by looking lengthwise through the tubes against a dull white background. Readings are first made

[7] The standard calcium oxalate solution is made by dissolving 0.0630 gm. of pure oxalic acid ($H_2C_2O_4.2H_2O$) in water. To this are added 200 cc. of N hydrochloric acid and sufficient calcium chloride solution to correspond to 20 mg. of calcium. The whole is made up to 1 liter with water. 1 cc. of this solution is equivalent to 0.02 mg. of calcium as calcium oxalate.

[8] The ferric thiocyanate solution is made from two solutions which are mixed ½ hour before use. Solution A is 0.3 per cent ammonium thiocyanate. Solution B is 0.3 per cent ferric chloride, made up from the salt with its contained water of crystallization, adding a few drops of acid, if necessary, to clear the solution. 5 cc. portions of Solutions A and B are mixed and the whole is diluted to 25 cc. with water. This is the proper dilution for use with ordinary serum, but when smaller amounts of calcium are present the solution should be more dilute; with larger amounts it should be more concentrated.

against standard oxalate solutions differing from each other by 0.5 cc., interpolating when necessary. Extra standards may then be made up to obtain more exact results.

The calculation is simple, as, for example, 2 cc. of serum were used, and a 6 cc. aliquot portion of the oxalate solution was taken. The color in the comparison tube, after adding thiocyanate and diluting to the mark, was found to match that corresponding to 6.3 cc. of the standard oxalate solution; then

$$6.3 \times .02 \times \frac{10}{6} \times \frac{100}{2} = 10.5 \text{ mg. per 100 cc. of serum.}$$

It is necessary to run blank determinations on the reagents used and to make allowance for any blank found to be present. In our experience we have never encountered a blank amounting to more than 0.1 cc. of the standard oxalate solution. Other workers, however, have informed us that some samples of nitric acid contain appreciable amounts of calcium. We have used Baker's analyzed nitric acid and have found it free from significant amounts of calcium.

Results of Calcium Determinations.

Solutions of calcium chloride containing known amounts of calcium, as determined gravimetrically, were suitably diluted, and portions containing amounts of calcium comparable to the amounts present in the serum used were analyzed according to the method described above. The range of accuracy on solutions containing from 0.1 to 0.2 mg. of calcium was usually within 2 per cent of the theoretical values. Exceptionally a variation of as much as 5 per cent from the theoretical value was encountered; the average of duplicate determinations, however, almost invariably agreed within 2 per cent of the theoretical.

Known amounts of calcium (0.1 to 0.2 mg.) added to 2 cc. samples of serum were quantitatively recovered with the same degree of accuracy as in pure solution.

The serum of normal adults, of infants, and of dogs was found to contain from 9.5 to 11.5 mg. of calcium per 100 cc. In certain pathological conditions, such as infantile tetany, and terminal uremic conditions, great reductions in the calcium content of the serum were observed.

Serum has been used instead of whole blood since it is known that the corpuscles contain no calcium, and that the fibrin clot contains only a very small and fairly constant amount of calcium. If whole blood were used the calcium content would vary inversely with the corpuscular count, other things being equal.

Technique of the Magnesium Method.

When it is desired to determine magnesium in the same sample of serum used for calcium determination, the procedure for calcium has to be modified somewhat. After precipitation of the calcium oxalate, as previously described, the contents of the beaker are rinsed into a conical centrifuge tube and centrifuged for about 10 minutes. The clear liquid is syphoned off[9] into a small casserole or a platinum dish and used for magnesium determination. The calcium oxalate remaining in the centrifuge tube is dissolved in 0.5 cc. of concentrated nitric acid. This is best accomplished by first adding the acid and then 5 or 10 cc. of water; the tube is then heated by immersion in a beaker of hot water and the contents are stirred by bubbling air through the tube by means of a capillary glass tube reaching to the bottom of the centrifuge tube and connected with a rubber bulb. The contents of the tube are then transferred to the beaker and evaporated to a volume of about 5 cc.; the calcium is then reprecipitated and determined exactly as described above.

The liquid syphoned off into the dish contains the magnesium[10] present, to this is added 0.5 cc. of concentrated sulfuric acid, and the liquid is evaporated to dryness on an electric stove. Ashing is completed over a Méker burner. If a small amount of black residue remains, a drop of sulfuric acid is added, after cooling, and the ashing repeated. To the residue is added 0.5 cc. of con-

[9] Syphoning is accomplished by means of a small glass tube, one end of which is drawn to a short capillary point and bent upwards. This form of tip prevents the sucking up of any precipitate and permits the removal of almost all of the liquid. The syphon tube is passed through a small double-bored rubber stopper, which fits the centrifuge tube. A short glass tube passes through the other hole in the stopper. The syphon is started by blowing into this tube or by the use of an atomizer bulb.

[10] Approximately 0.2 cc. of liquid remain in the centrifuge tube, and this amount, which is about 2 per cent of the total, may be allowed for in the final calculation, if extreme accuracy is desired.

centrated hydrochloric acid and a little water. The dish is warmed and the contents are transferred to a small (25 cc.) beaker with several washings of hot water. The beaker is put on an electric stove and the contents are evaporated to a volume of about 3 cc., a drop of phenolsulfonephthalein is added, and then 1 cc. of ammonium phosphate solution.[11] 1 cc. of concentrated ammonia is run in with stirring and the beaker allowed to stand over night. Crystals of ammonium magnesium phosphate separate out on the sides and bottom of the beaker.

The contents of the beaker are transferred to a conical 15 cc. centrifuge tube and centrifuged for a couple of minutes. The liquid is syphoned off in the manner above described and the precipitate in beaker and tube washed with 10 per cent ammonia (one part concentrated ammonia to nine parts of water), the precipitate in the tube being stirred up each time with the capillary blowing tube. It is unnecessary to dislodge the precipitate from the sides of the beaker. The washing is repeated four times, each time with from 6 to 10 cc. of ammonia. The precipitate is finally washed once with 95 per cent alcohol made just alkaline with ammonia. This is syphoned off and tube and beaker are dried in an air oven at about 60°C.

After drying, the contents of beaker and tube are dissolved in 10 cc. of 0.01 N hydrochloric acid and an aliquot portion is pipetted off for colorimetric determination.

The colorimetric comparison is done in the same way as in the calcium determination with the exception that the thiocyanate solution used is diluted to 40 or 50 cc. instead of to 25, a standard solution of magnesium ammonium phosphate[12] is used instead of

[11] Ammonium phosphate solution is made as follows: 25 gm. $(NH_4)_2PO_4$ are dissolved in 250 cc. H_2O. 25 cc. of concentrated ammonia are added and the mixture is allowed to stand over night. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled, and made up to 250 cc.

[12] This solution is made by dissolving 0.102 gm. of air-dried magnesium ammonium phosphate ($MgNH_4PO_4.6H_2O$) in 100 cc. of 0.1 N hydrochloric acid and diluting to 1 liter with water. Of this solution 1 cc. is equivalent to 0.01 mg. of magnesium. Magnesium ammonium phosphate loses water of crystallization when heated and must therefore be dried at room temperature. Commercial preparations of the salt are generally unreliable; it should be prepared by precipitation of pure solutions. See Jones, W., *J. Biol. Chem.*, 1916, xxv, 87.

one of calcium oxalate, and dilutions in the Nessler tubes are made with 0.01 N hydrochloric acid. The calculation of results is as follows: If 2 cc. of serum were used and a 6 cc. aliquot portion of the solution of the precipitate were taken, and the color found to be the same as that of 3.2 cc. of the standard magnesium solution, then

$$3.2 \times 0.01 \times \frac{10}{6} \times \frac{100}{2} = 2.66 \text{ mg. magnesium per 100 cc. of serum.}$$

Results of Magnesium Determinations.

Solutions containing known amounts of calcium and magnesium were analyzed as described above, and amounts of magnesium ranging from 0.02 to 0.04 mg. were recovered practically quantitatively; the average of duplicate determinations being within 5 per cent of the theoretical value. Similar amounts of magnesium added to serum were quantitatively recovered, the presence of organic matter having no effect on the accuracy of the determination. Human blood serum was found to contain from 2.2 to 3.5 mg. of magnesium per 100 cc.

On account of the extremely small amounts of magnesium present in the serum and the number of manipulations necessary, we feel that the method is hardly as accurate as the calcium method but that it is possible to obtain sufficiently accurate results for comparative purposes.

Detailed results of calcium and magnesium determinations in the serum of infants suffering from rickets and tetany will appear shortly in *The Quarterly Journal of Medicine.*

A MICRO METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHATES IN THE BLOOD SERUM.

By W. McKIM MARRIOTT and F. H. HAESSLER.

(From the Department of Pediatrics, Johns Hopkins University, Baltimore.)

(Received for publication, October 1, 1917.)

The method here described was devised in order to determine whether or not a retention of inorganic phosphates, with a resulting increase of these substances in the blood serum, occurs in patients suffering from certain types of nephritis. Results obtained by the use of the method in such cases have already appeared.[1]

The method depends upon the same principle as that for magnesium determination described in the preceding paper;[2] namely, the decolorization of solutions of ferric thiocyanate by an acid solution of ammonium magnesium phosphate. The phosphates of the serum are precipitated directly by means of magnesia mixture.

Technique of the Method.

Dilute 1 cc. of clear serum with about 5 cc. of water in a small beaker. Add 2 drops of 0.1 N hydrochloric acid and 1 cc. of magnesia mixture.[3] Then with stirring, run in slowly 2 cc. of 10 per cent ammonia (one part concentrated ammonia to nine parts of water), and allow to stand over night in a cool place.

The precipitate is filtered off on a small Gooch crucible, the mat being prepared as for the calcium determination.[2] Wash the

[1] A preliminary report of the method appeared in the Proceedings of the American Society of Biological Chemists, *J. Biol. Chem.*, 1916, xxiv, p. xviii. For results in nephritis cases see Marriott, W. McK., and Howland, J., *Arch. Int. Med.*, 1916, xviii, 708.

[2] Marriott and Howland, *J. Biol. Chem.*, 1917, xxxii, 233.

[3] Dissolve 10 gm. of magnesium chloride (sticks) and 5 gm. of ammonium chloride in 250 cc. of water; add 10 cc. of concentrated ammonia. Allow to stand over night, filter, neutralize with hydrochloric acid, using phenolsulfonephthalein as an indicator, and make up to 500 cc. with water.

precipitate and beaker four times, each time with about 5 cc. of the 10 per cent ammonia, then once with 95 per cent alcohol made just alkaline with ammonia, and finally with 5 cc. of ether. Suction is kept on for several minutes in order to dry the mat. The crucible is then returned to the beaker and 10 cc. of 0.01 N hydrochloric acid are run in and allowed to percolate through the crucible. The beaker is covered with a rubber dam or put in a desiccator over water and allowed to stand for a couple of hours or over night. The asbestos is then stirred up in the acid and the suspension transferred to a centrifuge tube and centrifuged. A 6 cc. aliquot portion of the clear liquid is pipetted off and used for colorimetric determination. This is carried out exactly as the magnesium determination, except that a solution of magnesium ammonium phosphate of a somewhat different strength is used as a standard.[4]

The calculation of results is as follows: If 1 cc. of serum were used and a 6 cc. aliquot portion of the dissolved precipitate taken for colorimetric determination, the color in the Nessler tube corresponding to that resulting from the addition of 0.9 cc. of the standard phosphate solution, then

$$0.9 \times 0.02 \times \frac{10}{6} \times \frac{100}{1} = \begin{matrix} 3.0 \text{ mg. phosphorus} \\ \text{per 100 gm. of serum.} \end{matrix}$$

RESULTS.

Solutions of inorganic phosphates containing from 0.01 to 0.1 mg. of phosphorus were analyzed according to the method described and quantitative results obtained. Similar amounts of phosphate added to the serum were quantitatively recovered, the presence of organic matter apparently having no effect in preventing the precipitation of the phosphate. We further found that lecithin in amounts comparable to that occurring in serum, yields no inorganic phosphate when treated according to the method described.

[4] This solution is made by dissolving 0.1584 gm. of air-dried magnesium ammonium phosphate ($MgNH_4PO_4.6H_2O$) in 100 cc. of 0.1 N hydrochloric acid and diluting to 1 liter with water. 1 cc. of this solution is equivalent to 0.02 mg. of phosphorus.

The method is not applicable to whole blood, without modification, as the corpuscular debris renders filtration extremely slow. In some instances, the serum, if allowed to stand for too long a time before filtration, filters very slowly. In such instances the liquid may be centrifuged, the precipitate dissolved in dilute acid, and the phosphates reprecipitated.

Applying the method to the determination of inorganic phosphates in the serum of normal individuals we have found from 1.0 to 3.5 mg. per 100 cc. of serum. In the serum of nephritics suffering from acidosis amounts of inorganic phosphorus as high as 20 mg. per 100 cc. of serum have been determined.

IRON AS AN ANTIDOTE TO COTTONSEED MEAL INJURY.*

By W. A. WITHERS and FRANK E. CARRUTH.

(From the Chemical Division of the North Carolina Agricultural Experiment Station, West Raleigh.)

(Received for publication, September 5, 1917.)

As a result of feeding rabbits[1] successfully 106 days on cottonseed meal with ferric ammonium citrate, several experiments were conducted with pigs, using iron salts to prevent cottonseed meal poisoning. It was desired to learn whether cottonseed meal could be used safely in practice as a pig feed. These experiments are incomplete from a practical standpoint. They are of interest, however, as they relate to the question whether cottonseed meal injury is due to the presence of a harmful substance, or whether, as is claimed by some, it is due to inadequate diets or erroneous methods of feeding. It is believed that by using iron salts with cottonseed meal the toxic factor is more or less counteracted, thus allowing the diet to approach its true feeding value.

Although the addition of iron salts may not be recommended as a successful way for the practical feeder to overcome cottonseed meal poisoning, the experiments show that with iron salts much

* The feeding experiments with swine were conducted by the Animal Husbandry Division under the supervision of R. S. Curtis and later, D. T. Gray, Chief of the Division. The postmortem examinations were made by Dr. G. A. Roberts, Veterinarian of the Experiment Station. His observations are recorded in the *35th Ann. Rep., North Carolina Agric. Exp. Station*, 1911–12, 27.

[1] Rabbits are usually very quickly affected by cottonseed meal, even though long cooked. They are made sick in 1 to 4 weeks, depending on the toxicity of the meal. The experiments with rabbits were described by Withers and Brewster (*J. Biol. Chem.*, 1913, xv, 161). Regarding the rabbits receiving the iron salts, Withers and Brewster state: "Each of these rabbits was alive and normal after 58 days' feeding, each having consumed 870 gm.of cottonseed meal and 19.95 gm. of iron salts."

larger quantities of cottonseed meal have been fed and better gains made[2] than would have been possible otherwise, and that in many cases death has been averted.

Further evidence of the presence of a toxic substance in the meal is the fact that extraction with alcoholic alkali removes the toxicity (rabbit experiments[3]).

We have assumed that this is due to the formation of an insoluble iron salt of gossypol or one of its derivatives together with the catalytic acceleration of the oxidation of gossypol and perhaps a tonic action of iron on the system. Possibly with a liberal supply of iron, the blood of the animal is better enabled to convert gossypol, in whatever form absorbed, into non-toxic products This action of iron salts has no adequate explanation from the deficiency standpoint. Treatment of cottonseed meal with iron salts causes the meal to turn brownish black, indicating a reaction with some component of the meal. A similar color may be produced by the action of iron salts on a solution of gossypol.

EXPERIMENTAL.

Three lots of six pigs each were fed cottonseed meal with corn meal, 1:3, and two other lots of three each were fed equal parts of cottonseed meal and corn meal. It was planned to feed cottonseed meal to the extent of 1 per cent body weight daily in the first three lots, and 2 per cent daily in the other lots, with a correspondingly large amount of iron salt. As the experiment progressed the amounts fed fell below the 1 per cent and 2 per cent basis.

The pigs were kept in dry lots about 50 by 100 feet. The conditions were much more favorable than in the experiments to be described later, in which pigs were confined in pens under a barn during the winter.

The percentage composition of the diets was as follows:

Lot No.	243	239	242	258	259
Cottonseed meal	25	25	25	50	50
Corn meal	75	75	75	50	50

[2] This gain, of course, is due to the larger intake of food. Up to the time pigs are affected by cottonseed meal the gains are often not greatly different from those receiving iron salts.

[3] Withers, W. A., and Ray, B. J., *Science*. 1912, xxxvi, 31.

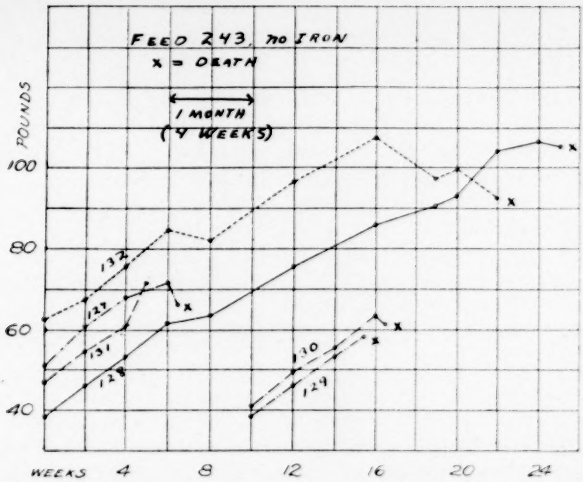

CHART I. Control lot. Six pigs on a diet of cottonseed meal and corn meal without iron all died before the experiment ended. Four deaths were typical sudden deaths.

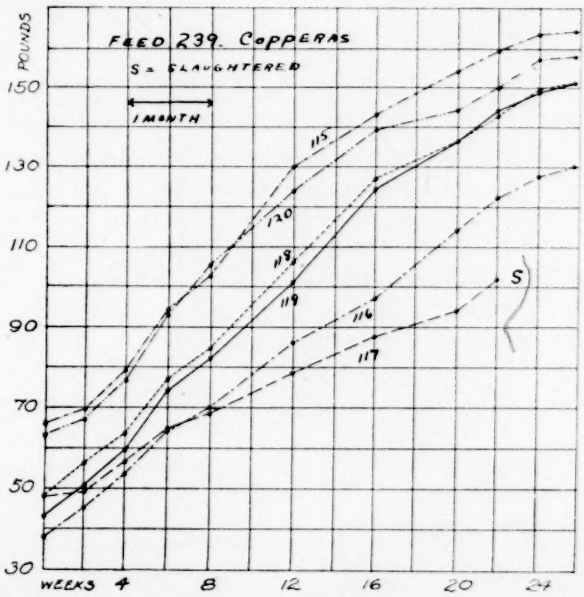

CHART II. Cottonseed meal with corn meal (1:3) with copperas solution. Five of the six pigs finished the 180 day experiment. The other was slaughtered after 154 days. No symptoms of cottonseed meal poisoning were evident on postmortem examination.

Lot 243 received to each 4 pounds of feed 1 gallon of water.

Lot 239 received to each 4 pounds of feed 1 gallon of a copperas solution (1 pound $FeSO_4.7H_2O$ dissolved in 50 gallons of water). 1 gallon contained approximately 9 gm. of copperas, or 2.4 gm. of iron.

Lot 242 received a similar solution containing approximately the same weight of $FeCl_3.6H_2O$.

Lot 258, three pregnant sows, received 1 gallon of a copperas solution four times as strong as that used in Lot 239. This furnished twice as much iron to each pound of cottonseed meal.

Lot 259, three pregnant sows, received a solution of $FeCl_3.6H_2O$ of similar strength.

These sows except Nos. 133 and 136 farrowed between the 10th and 12th weeks. Up to this time they made excellent gains and after farrowing steadily fell off. Below are given the daily gain up to the 63rd day and the other data regarding the young.

Sow No.	134	135	136	137	138
Daily gain (63 days)	0.74	0.79	0.87	0.71	0.82
Number of young born	2	5	0	3	4
Number of young living 3 weeks or more	2	4	0	2	4

These results with iron salts in connection with cottonseed meal have an interesting application in regard to the theory of nutritive deficiencies of cottonseed meal as fed in these diets. The toxic factor, being more or less completely inhibited by the iron salt, leaves any deficiency of the diet to show its effect on the growth. In other words, the results of the copperas experiment show that by eliminating the toxic factor we have no evidence of nutritive deficiency in cottonseed meal great enough to produce failure in a relatively short period.

The toxic factor is probably not so well overcome here as in subsequent experiments in which cottonseed *kernels* were extracted by ether and fed.

The growth curves for these experiments are shown in Charts I, II, III, and IV.

Experiment 2.—All the pigs which survived the previous 180 day experiment, together with some shoats of about 100 pounds' weight, were started immediately at the close of the first experiment on a second experiment to test the value of iron salts. This experiment continued 121 days. It will be seen that in some cases pigs were fed on cottonseed meal for a total of 301 days.

TABLE I.

Experiment 1.

Pig No.	Weight.				Result.	Duration.
	Initial.	Final.	Daily gain.	Cotton-seed meal eaten.		
Feed 243. Check lot. No iron.						
	lbs.	*lbs.*	*lbs.*	*lbs.*		*days*
127	51	66	0.33	25	Died.	46
128	38.5	105	0.37	79	"	177
129	38.5	58	0.50	19.5	"	38
130	41	61.5	0.44	22	"	45
131	47	72	0.71	18	"	35
132	62.5	92.5	0.19	109.5	"	154
Average.	46.4	75.8	0.35	45.5		83
Feed 239. Copperas lot.						
115	66	164	0.54	128	Discontinued.	180
116	38	130	0.51	128	"	180
117	48	101.5	0.35	71	Slaughtered.	154
118	48	151	0.57	128	Discontinued.	180
119	43	151	0.60	128	"	180
120	63	158	0.53	128	"	180
Average.	51	142.6	0.52	118		176
Feed 242. Ferric chloride lot.						
121	63	163	0.56	128	Discontinued.	180
122	41	126.5	0.46	128	"	180
123	33	63.5	0.49	31.5	Died.	62
124	35	73	0.25	23	Slaughtered.	154
125	43.5	123.5	0.48	128	Died.	167
126	53	162	0.61	128	Discontinued.	180
Average.	44.7	118.6	0.48	94.4		154
Two other lots of sows were fed 152 days on equal parts of cottonseed meal and corn meal.						
Feed 258. Stronger copperas solution.						
133	85	110	0.51	50.5	Died.	44
134	98	113.5	0.10	228	Discontinued.	152
135	98	117	0.13	228	"	152
Average.	93.7	113.5	0.17	169		116
Feed 259. Stronger ferric chloride solution.						
136	82.5	171	0.51	228	Discontinued.	152
137	92.5	120	0.18	228	"	152
138	84	139	0.36	228	"	152
Average.	86.3	143.3	0.37	228		

The ration was made up as before of 25 per cent cottonseed meal and 75 per cent corn meal. The various lots received in addition:

Feed 270. Control.
" 271. Hardwood ashes (pigs had access to boxes containing ashes) (copperas first 2 weeks).
" 272. Copperas solution and ashes as above.
" 273. Copperas solution.

The pigs were fed from troughs in large outdoor lots. The animals rooted considerably. The control lot consisted of fresh shoats which were fed until sickness occurred in all and then the survivors were transferred to the other lots. The data of the experiments are summarized in Tables II and III.

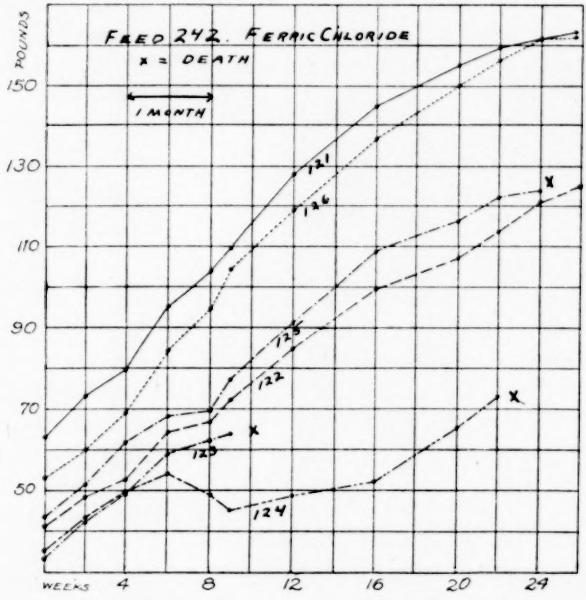

CHART III. Cottonseed meal and corn meal (1:3) with ferric chloride do not here seem as efficient as with copperas, but there is marked improvement over the control lot.

In some of these lots, especially in Lot 273, no conclusion regarding iron or ashes as antidotes may be drawn. The deaths in Lot 273 were probably due to results of the previous feeding. In all lots except No. 270 the meal was fed considerably below the basis of 1 per cent body weight. These animals were also much older than those of other experiments.

TABLE II.

Pig No.	Weight.				Result.	Duration.
	Initial.	Final.	Daily gain.	Cotton-seed meal eaten.*		
Feed 270. No antidote.						
	lbs.	*lbs.*	*lbs.*	*lbs.*		*days*
139	98	115	0.38	45	Died.	45
140	104	130	0.53	49	Transferred.	49
142	106	145	0.80	49	"	49
144	102	140	0.78	49	"	49
Average.	102.5	132.5	0.62	48		48
Feed 271. Ashes.						
120	158	225	0.55	121	Slaughtered.	121
121	163	260	0.80	121	Kept for breeding.	121
135	117	190	0.69	121	Slaughtered.	121
138	139	230	0.75	121	"	121
141	105	185	0.66	121	"	121
140	130	150	0.28	72	"	72
Average except 140.	136.4	218	0.67	121		
Feed 272. Copperas and ashes.						
115	164	275	0.92	121	Slaughtered.	121
118	151	205	0.45	121	"	121
122	126.5	190	0.52	121	"	121
136	171	245	0.61	121	"	121
143	100	165	0.54	121	"	121
142	145	200	0.46	72	"	72
Average except 142.	142.5	216	0.61			
Feed 273. Copperas.						
116	130	115	Lost.	119.5	Slaughtered.	121
119	151	152	0.13	7	Died.	8
126	162	285	1.02	119.5	Slaughtered.	121
134	113.5	195	0.67	119.5	"	121
137	125	140	0.26	54.5	Died.	57
144	140	142	0.29	7	"	7

* Estimated from weight of pigs and amounts fed to each lot.

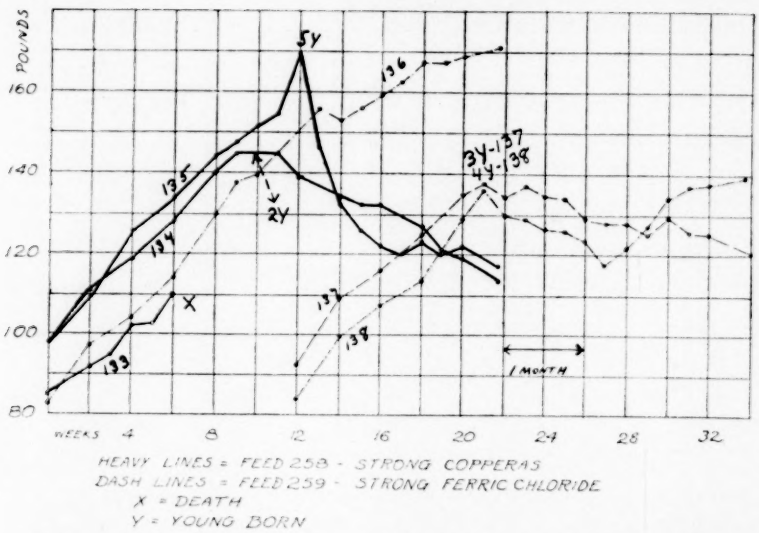

CHART IV. Pregnant sows were fed on a diet containing equal parts of corn meal and cottonseed meal. The iron solutions used on Feeds 258 and 259 furnished twice as much iron per pound of cottonseed meal as was used in Feeds 239 and 242. Disregarding the one early death these sows made excellent gains until farrowing time,—gains which one would not expect on a diet markedly lacking in one or more dietary factors.

It is of interest to compare the gains of the pigs which were also on the previous experiment and were in some cases, therefore, fed cottonseed meal 301 days consecutively.

Experiment 3.—In another experiment with four lots of nine pigs each, the pigs were confined in pens under a barn. Iron salts did not appear to be so advantageous, yet the deaths were considerably fewer among those pigs which received iron salts.

The experiment continued 94 days, after which the surviving pigs were slaughtered. The rate of feeding was practically the same—4 per cent of weight daily at the start—but this was not maintained. The grain ration in all cases was the same (cottonseed meal 25 per cent and corn meal 75 per cent) with the variations here noted. The lots received to each 4 pounds of feed:

Feed 332. 1 gallon of water.
" 333. 1 " " copperas solution.
" 334. 1 " " ferric chloride solution.
" 335. 1 " " water with hardwood ashes supplied *ad libitum* as before.

TABLE III.

Pig No.	Experiment 1.	Experiment 2.	Weight.			Total days fed.
			Initial.	Final.	Daily gain.	
			lbs.	*lbs.*	*lbs.*	
120	Copperas.	Ashes.	63	225	0.54	301
121	$FeCl_3$.	"	63	260	0.65	301
135	Stronger copperas.	"	98	190	0.30	301
138	Stronger $FeCl_3$.	"	84	230	0.48	301
115	Copperas.	Copperas and ashes.	66	275	0.69	301
118	"	"	48	205	0.52	301
122	$FeCl_3$.	"	41	190	0.49	301
136	Stronger $FeCl_3$.	"	82.5	245	0.54	301
116	Copperas.	Copperas.	38	115	0.27	301
119	"	"	43	152	0.58	188*
126	$FeCl_3$.	"	53	285	0.77	301
134	Stronger copperas.	"	98	195	0.32	301
137	Stronger $FeCl_3$.	"	92.5	140	0.20	237*

* Died.

Feed 332, control, was fatal to six pigs out of nine. Deaths occurred on the 36th, 40th, 43rd, 44th, 53rd, and 59th days.

Feed 333, copperas, was fatal to three pigs out of nine. Deaths occurred on the 40th, 41st, and 58th days.

Feed 334, ferric chloride, was fatal in only one case,—on the 59th day.

Feed 335, hardwood ashes, was fatal to seven pigs out of nine. Deaths occurred on the following days: 46th (2), 47th (2), 48th (2), and 59th.

Thus of a total of eighteen pigs receiving iron, only four died, while of a total of eighteen pigs without iron, thirteen deaths occurred.

These four deaths of pigs receiving iron showed that the mere addition of iron was not sufficient to overcome the toxic effects and deficiencies of this diet under such severe conditions.

Experiment 3 indicates clearly that the iron has some antidotal action.

TABLE IV.

Experiment 3.

Pig No.	Weight.					Remarks.
	Initial.	Maximum.*	Final.	Daily gain or loss.	Cottonseed meal eaten.†	
	Feed 332. No iron salt added.					
	lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*	
49	148.5	171	149	0.01	84	Lived.
50	123	138	104	−0.36	53	Died, 53 days.
65	115	136	125	0.11	66	Lived.
66	124	143	139	0.17	71	"
67	78	85	78	0.00	27	Died, 43 days.
68	96.5	108	107	0.18	44	" 59 "
69	75	85	85	0.23	27	" 44 "
70	81	82	76	−0.14	25	" 36 "
71	89.5	98	98	0.25	31	" 40 "
Average.	103.4	116	107	0.05	47	
	Feed 333. Copperas.					
72	95.5	152.5	133.5	0.40	82	Lived.
73	125.5		223.5	1.05	105	"
74	100	166.5	165	0.69	88	"
75	101.5	146.5	134.5	0.35	83	"
76	123		157	0.59	65	Died, 58 days.
77	68		104	0.38	56	Lived.
78	70.5		80	0.24	27	Died, 40 days.
79	67		85	0.44	26	" 41 "
80	78		129	0.54	65	Lived.
Average.	92.1		134.6	0.54	66	
	Feed 334. Ferric chloride.					
81	136		241	1.12	112	Lived.
82	162		259	1.03	130	"
83	120		188	0.72	97	"
84	104		145	0.70	52	Died, 59 days.
85	85.5		129	0.46	68	Lived.
86	65		115	0.52	56	"
87	95		156	0.65	77	"
88	90		127	0.39	72	"
89	62		109	0.50	52	"
Average.	102.2		163.2	0.67	80	

TABLE IV—*Concluded.*

Pig No.	Weight.					Remarks.
	Initial.	Maximum.*	Final.	Daily gain or loss.	Cottonseed meal eaten.†	
	Feed 335. Hardwood ashes *ad libitum*.					
	lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*	
90	144		190	1.00	59	Died, 46 days.
91	138.5		202.5	0.68	94	Lived.
92	93		136	0.92	39	Died, 47 days.
93	123		180	0.61	83	Lived.
94	135	178	176	0.69	64	Died, 59 days.
95	65		90	0.54	26	" 46 "
96	48.5		74	0.54	20	" 47 "
97	58.5		83	0.51	25	" 48 "
98	84		108	0.50	36	" 48 "
Average.	98.8		137.7	0.66	50	

* The maximum weights in this table are given when they exceed the final weights.

† Estimated from feed given, body weight of pig, and number of days.

Summary.

Feed.		Weight.				Deaths.
		Initial.	Final.	Daily gain.	Cottonseed meal eaten.	
		lbs.	*lbs.*	*lbs.*	*lbs.*	
332	No iron.	103.4	107	0.05	47	6
333	Copperas.	92.1	134.6	0.46	66	3
334	Ferric chloride.	102.2	163.2	0.68	80	1
335	Hardwood ashes.	98.3	137.7	0.67	50	7

Experiment 4.—A fourth experiment with iron salts was conducted under the indoor conditions of the previous experiment. The pigs were of a rather poor quality, which may account for the small gains made. Four lots of seven pigs each were fed on the following rations in the form of a slop:

Feed 342. Cottonseed meal 1 part plus cracked corn 7 parts,—control.
" 343. The same plus copperas solution.
" 344. The same plus iron-ammonium citrate solution.
" 345. Cottonseed meal 1 part, wheat bran 3 parts, cracked corn 4 parts.
" 346. Peanut meal 1 part and cracked corn 7 parts.

This experiment continued from November 29 to April 3 (127 days). There was one death in the control lot (Feed 342) in the 1st week and two in the copperas lot (Feed 343) on the 74th day and the 124th day. Disregarding these three pigs the average gains were best in the following order: (1) Iron-ammonium citrate. (2) Copperas. (3) Peanut meal. (4) Straight cottonseed meal. (5) Wheat bran.

TABLE V.

Feed.		Average weight.			Average gain.
		Initial.	Final.	Daily gain.	
342	Check.	55.5 (6 pigs.)	86.7	0.25	31.2
343	Copperas.	50.0 (5 ")	95.4	0.36	45.4 (42.1)*
344	$FeCl_3$.	50.7	105.1	0.43	54.4
345	Wheat bran.	52.8	90.8	0.30	38.0
346	Peanut meal control.	51.6	92.0	0.31	40.3

* Including the gain of the two pigs that died.

All these pigs were in extremely poor condition at the end of the experiment. The gains were slight and the animals were found to be badly infested with internal and external parasites. Postmortem examination showed that practically all the animals were in a pathological condition.

Several experiments conducted at other stations have also indicated that iron salts have an antidotal action, and private individuals have also reported a beneficial action by copperas.[4] It is apparent that where our experiments were conducted under very unfavorable conditions, the salutary effect of the iron salts is not so pronounced as under favorable conditions.

SUMMARY AND CONCLUSIONS.

1. Four feeding experiments with pigs have shown that iron salts have a decidedly beneficial action in preventing cottonseed meal injury. Much larger quantities of meal are consumed, deaths have been postponed or averted, and better gains have been made when an iron salt is added to the feed.

[4] Walker, G. B., *Mississippi Agric. Exp. Station Bull. 177*, 1916. Wells, C. A., and Ewing, P. V., *Georgia Agric. Exp. Station Bull. 119*, 1916; also several personal communications.

2. Wood ashes apparently have no antidotal action in averting death but as the lot receiving ashes made much better gains it is possible that this is due to improvement of the inorganic part of the diet composed of corn and cottonseed meal.

3. The suggestion is made that the iron salts combine with, or facilitate oxidation of the harmful substances in cottonseed meal.

4. Iron salts have an antidotal action towards cottonseed meal poisoning of rabbits and swine.

5. By thus controlling the toxic factor, it is shown that cottonseed meal injury is not due to a lack of "vitamines" or to deficiencies in calcium, sodium, and chlorine,—which ash analyses might lead one to suspect as the limiting mineral factors in a diet of cottonseed meal and corn.

SOME FACTORS INFLUENCING THE RESPIRATION OF GROUND NERVOUS TISSUE.

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We have undertaken to make a systematic study of respiration of the central nervous system. Distilled water, in spite of several disadvantages, was chosen as the medium, because by its use the smallest number of unknown influences are introduced. Physiological salt solutions did not give larger values than water. 1 per cent glucose, 1 per cent peptone, or 1 per cent lecithin solutions each gave less respiration than water. Blood as a medium for brain gives larger values than water or physiological salt solutions. But these larger values are partly due to the respiration of the blood itself, probably partly also to the catalytic action of the hemoglobin. The amount of oxidation in a slightly alkaline solution[1] is considerably larger than in water; but it is well known that dilute alkali is favorable for the oxidation of many tissue substances not necessarily related to respiration.

This report attempts to answer the following questions: Can a satisfactory extract of respiring substances be obtained? Does carbon dioxide or oxygen storage affect values obtained? Were the results altered by bacterial action? What is the temperature coefficient of that part of the respiration that is comparatively stable in water medium? Do blood constituents materially alter the amount of respiration obtained? How soon after the death of an animal does the rate become constant? How does brain respiration compare with that of other more carefully studied tissues in the same animal? Are there differences in the rate of oxidation in various divisions of the central nervous system? Are

[1] Batelli, F., and Stern, L., *Biochem. Z.*, 1909, xxi, 487.

there consistent differences in white and gray matter? Does the age of the brain affect the respiration in the same way as age affects the total metabolism of the animal? And, finally, how do various adult animals differ from each other?

The method used was similar to one that had given rather satisfactory results in a study of the rate of oxidation of several easily oxidizable compounds.[2]

Apparatus.

The *small tube* used in the following experiments is the Bunzel oxidase tube.[3] It consists of an inverted Y-shaped test-tube with a ground glass stopper carrying a capillary manometer graduated in millimeters. The tube holds about 25 cc. When used in this investigation a 3 cc. test-tube containing 1 cc. of 0.5 N sodium hydroxide was inserted in the upper part after the tissue mixture had been introduced.

When ready for an experiment the small tubes were placed in a shallow perforated wooden box and clamped to it. This box holds eighteen small tubes. It was fastened to a mechanical shaker run by an electric motor. The shaker is in an air thermostat regulated to about 0.2°C.

Data for Calculation of Results.

The small tubes held 25 cc. In these there were placed 3 gm. of tissue, 6 cc. of H_2O, and 1 cc. of 0.5 N NaOH (in the inner 3 cc. test-tube). There were, therefore, approximately 15 cc. of space to be occupied by air, of which about 3 cc. would be oxygen. The atmospheric pressure averaged 750 mm. of mercury. 1 cc. of the gas in the tube corresponds to 50 mm. of mercury, and 1 mm. of mercury in manometer tubes represents 0.02 cc. of oxygen or 0.000024 gm. 0.02 cc. at 37° becomes 0.018 cc. at 0°.

The 1 cc. of 0.5 N NaOH was carefully measured with a pipette. At the end of the experiment the carbon dioxide present was determined by titrating the contents of the *3 cc. tube* with 0.02

[2] Mathews, A. P., *J. Biol. Chem.*, 1909, vi. 3. MacArthur, C. G., *J. Physic. Chem.*, 1916, xx, 545.

[3] Bunzel, H. H., *J. Biol. Chem.*, 1914, xvii, 409.

N H_2SO_4 using phenolphthalein as an indicator first, and then titrating to end-point with Congo red. The number of cc. taken to neutralize the solution to the Congo red end-point after neutralization to phenolphthalein approximately represents the amount of carbon dioxide present. 1 cc. of an hypothetical 0.02 N carbon dioxide solution would have 0.00044 gm., or 0.223 cc. of CO_2 in it. The number of cc. used for the Congo red titration as indicated above, multiplied by 0.00044 gives the weight of CO_2 liberated from the tissue in the given period of time; or if multiplied by 0.223, it gives the volume of CO_2 at 0°; and if by 0.253, the volume at 37°.

In the following tables the figures in the last two columns are corrected to 0°C. and 760 mm. of mercury.

EXPERIMENTAL.

Tissue and Medium.—At first it was thought possible to use a solution of the respiring substances. If this could be done the technique would be greatly simplified and several sources of error would be eliminated. Centrifuging a water extract of the ground brain was tried repeatedly. The nature of the extracting medium was altered, also the fineness of grinding the tissue, the length of extracting time, and the length of time the water tissue was centrifuged. In all cases no distinct separation of the respiring substance could be obtained.

It was found that only a part of the respiring compounds go into solution. The amount varied considerably in the various experiments, depending on the length of time centrifuged and the time the tissue stood before centrifuging. It is entirely possible, that the rough separation of about two-thirds in the residue and one-third in the solution may indicate the comparative amounts of oxydones and oxidases present.[4]

Filtering through several layers of cheese-cloth gives milky solutions of widely different oxidative power. Filtering through paper was so slow and so incomplete and the changes in the medium were so large that it was deemed wholly unsatisfactory.

Because of unsatisfactory results with the centrifuging of the original hashed material, grinding the tissue with sand, glass, or

[4] Batelli and Stern, *Compt. rend. Soc. biol.*, 1913, lxxiv, 212.

Kieselguhr and centrifuging the water extract were tried. It was found that if the grinding was thorough but little respiration occurred.[5] This may be due to the destruction of the cells, or the sand may absorb one or more of the respiring substances, or the disintegration may allow cell localized substances to interact to prevent respiration.

Because of this lowering of respiration due to grinding the brain tissue in sand, it was of course necessary to show that the grinding with the meat grinder did not materially affect the results. In several experiments with tissue varying in size from that of the grinder (small sized grating used) to about one-fourth inch cubes, practically no differences in either oxygen consumed or carbon dioxide produced were found. It was therefore decided to use in this work the tissue ground with an ordinary meat grinder. However, the possibility of separating the compounds causing respiration is a very interesting one and is being studied more in detail.

O and CO_2 Storage.—It would increase the reliability of the results if it were known that the results obtained represented the amount of O used and CO_2 produced during the time of the experiment, and were not partially due to O and CO_2 in the tissue at the beginning of the experiment. To obtain some information about this point 20 per cent H_2SO_4 was used as a medium for the tissue in comparison with a water medium. It was hoped that this strong acid would stop the respiration and also drive out all the O and CO_2 in the tissue. It is believed that the results indicate this to be true.

TABLE I.

Calf Brain. O Reading 2 Hrs. after Death.

Time in hrs.	Hg. by manometer readings.			Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
	1	2	3				
	mm.	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
Tissue in H_2O	16	28	38	0.76	2.3	0.072	0.048
" " "	17	30	40	0.80	2.5	0.076	0.053
" " H_2SO_4	6	9	10	0.20	0.5	0.019	0.010
" " "	5	7	8	0.16	0.4	0.015	0.008

[5] Harden, A., and Maclean, H., *J. Physiol.*, 1911–12, xliii, 34. Batelli and Stern, *Compt. rend. Soc. biol.*, 1914, lxxvi, 575.

Roughly speaking the amount of oxygen and carbon dioxide found in the tissues is between 15 and 20 per cent, of the total after 3 hours' shaking. It is possible, of course, that there is some respiration even in the presence of the strong acid. This is probably not large at any rate, because the results indicate that nearly all the oxygen consumed takes place in the 1st hour, whereas in respiration it continues throughout the experiment.

Two other flasks containing tissue and water were run along with the one in the above table. After the experiment strong H_2SO_4 was added to these flasks and shaken an hour longer to see how much CO_2 was left in the tissue after the 3 hour period. About the same amount remained in the tissue after the experiment as was found in the tissue at the beginning. Though the data do not give conclusive evidence, there seemed to be no reason for correcting the values directly obtained, either for oxygen or carbon dioxide.[6]

Bacterial Action.—Of course it was necessary to consider the possibility that the results were all due to bacterial action. There are several reasons for not believing so. First, though there is considerable variation in check experiments or in successive experiments, there is much greater uniformity and predictability than there would be were bacteria the cause. Second, it is well known that for some time following death bacteria get a foothold on a tissue with considerable difficulty, indicating protective substances. Third, if bacteria were the cause, one would expect that there would be much more oxygen consumed and carbon dioxide produced the last hour than the 1st. From the data it will be seen that as a rule there is a little more respiration the 1st hour. Lastly, it was found experimentally that following the orthodox aseptic practice in removing the brain, treatment of instruments, apparatus, etc., brought practically the same results as our regular technique. We obtained results after allowing the tissue to stand 8 hours in the ice box and then experimenting with it at 37° for 3 hours which showed that bacterial action had been very appreciable. It is believed that under the conditions of experimentation when the tissue is placed in tubes within 2 hours

[6] Harden and Maclean, *J. Physiol.*, 1911–12, xliii, 34.

and is not kept at 37° more than 3 hours practically no bacterial action occurs.[7]

Effect of Heating to 100°.—It was also desirable to know whether heat stopped the respiration. The effect of boiling 3 gm. of the tissue plus the 6 cc. of water to be used in the tubes was the only heat factor studied.

TABLE II.

Time in hrs	Hg. by manometer readings. 1	2	3	Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
Old pig brain. O reading 3 hrs. after death.							
	mm.	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
Normal	8	18	25	0.50	3.1	0.048	0.065
Boiled 10 min.	3	6	9	0.18	1.5	0.017	0.032
Old dog brain. O reading 3 hrs. after death.							
Normal	15	30	40	0.80	3.5	0.076	0.073
Boiled 10 min.	8	15	24	0.48	1.5	0.046	0.032

Though there is a rather wide variation in the results, it is clear that heating does not completely destroy either the oxygen consumption or the carbon dioxide production. Between a third and a half of the original respiration continues after thorough heating. This would indicate that this part of the respiration is different from the rest and that the substances producing it are more stable, not of an enzymic character. The other half is more unstable and resembles, to a certain extent at least, enzymes.[1]

Toluene produced almost identically the same results as heat. It seemed probable that toluene altered the same substances in the same way as heat. Because of the susceptibility of the respiration to such substances they could not be used to preserve the tissue.

Temperature Coefficient.—With an increase of 10° in temperature there should be approximately a doubling in rate of reaction, if the process is a chemical one.

The following experiments at 37° were run in the air thermostat above mentioned. For those at 25° a water thermostat was used in which a brass mechanical shaker was operated at the same rate

[7] Tashiro, S., *Am. J. Physiol.*, 1913, xxxii, 117.

as the one in the air thermostat. In the water thermostat the tubes up to the manometer portion were continually immersed in water. The reason for using both shakers was that the two temperatures could be compared on the same sample of tissue.

TABLE III.

Time in hrs.	Hg. by manometer readings. ½	1	2	3	Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
Old pig brain. O reading 3 hrs. after death.								
°C	*mm.*	*mm.*	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
37	10	15	22	33	0.66	3.2	0.063	0.067
	8	12	18	28	0.56	3.1	0.053	0.065
	7	13	20	30	0.60	3.3	0.057	0.069
25	5	8	12	16	0.32	1.7	0.031	0.036
	4	8	12	16	0.32	1.5	0.031	0.032
Old dog brain. O reading 50 min. after death.								
37	11	20	32	46	0.92	3.5	0.087	0.074
	11	20	34	48	0.96	3.4	0.091	0.071
	13	22	36	50	1.00	3.6	0.095	0.076
25	6	10	15	30	0.60	2.0	0.057	0.042
	4	7	12	27	0.54	1.7	0.051	0.036
	4	8	13	28	0.56	1.9	0.053	0.040

If one compares the amount of oxygen consumed at the end of an hour it will be seen that the temperature coefficient is about 2.2, while at the end of 3 hours it is not far from 1.8. The temperature coefficient for carbon dioxide production is about 1.8.

There is very little doubt that the comparatively low temperature coefficient is correlated with the nature of the respiration in nerve and brain. At present it is difficult to say what kind of an oxidation would have so low a temperature coefficient.[8] It may be that the explanation does not depend on a different kind of oxidation, but on the presence of temperature control substances that affect the rate more at higher temperatures than at lower.

Effect of the Blood in Tissue.—Though the portions of the brain used were freed carefully from blood, there was a very small amount present. To see what the rate of clot-free blood was

[8] Maxwell, S. S., *J. Biol. Chem.*, 1907, iii, 359.

and to see whether it had an accelerating action or simply an additive effect on tissue, the following experiments were undertaken. 6 gm. of clot-free blood plus 3 cc. of H_2O were used in one set of tubes; in the blood and brain set 6 gm. of blood and 3 of brain were used.

TABLE IV.

Sheep Brain. O Reading 1½ Hrs. after Death.

Time in hrs.	Hg. by manometer readings. ½	1	2	3	Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
	mm.	*mm.*	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
Normal tissue	11	19	42	57	1.14	4.2	1.08	0.088
" "	11	20	42	60	1.20	4.1	1.14	0.086
Blood	2	5	8	20	0.40	2.4	0.038	0.050
"	2	5	7	18	0.36	2.0	0.034	0.042
Blood and brain	15	30	50	72	1.44	5.5	0.136	0.115
" " "	16	35	52	75	1.50	5.7	0.142	0.120

Other experiments substantiate the above results, indicating rather strongly that the effect of blood is largely additive. Therefore the small amount of blood present in the brain used would produce a very negligible effect on the respiration.

Effect of Standing on Respiration.—Another point that needed to be studied was the effect on the oxygen consumption and carbon dioxide production of allowing the tissue to stand.

In the following experiment an old dog was killed, the brain removed and ground, 3 gm. with 6 cc. of water were placed in the tubes, and the small test-tube containing 1 cc. of 0.5 N NaOH was inserted. In the experiment recorded below this occupied 12 minutes. After the tubes were placed in the thermostat 10 minutes were allowed for the contents to come to the temperature of the thermostat. This length of time had previously been found to be sufficient if the tissue, which remained nearly the temperature of the animal, was added to water that had been heated to 37°C. Thus the O reading on the first set of tubes was obtained 22 minutes after the death of the animal. The second set was started 1 hour later than the first, the third set 3 hours after the first, and the last set 5 hours after. Up to the time of its use for the experiment the tissue was kept in the ice box.

TABLE V.

Old Dog Brain. O Reading 22 Min. after Death.

Time in hrs.	Hg. by manometer readings. ½	1	2	3	Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
	mm.	*mm.*	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
0	8	16	28	38	0.76	3.2	0.072	0.067
	9	16	28	38	0.76	3.5	0.072	0.074
1	5	14	22	28	0.56	2.8	0.053	0.059
	4	10	28	(30)	0.60	2.5	0.057	0.053
3	4	14	24	32	0.64	2.5	0.061	0.053
	5	16	24	29	0.58	2.5	0.055	0.053
5	3	9	17	28	0.56	2.5	0.053	0.053
	4	8	17	29	0.58	2.4	0.055	0.051

There seems to be considerably less respiration in the tissue after an hour of standing in the ice box than there is soon after death. The data would indicate that there is a rather rapid decrease in the respiration following death, but that it soon becomes nearly constant probably not far below the normal rate. After the preliminary rapid falling off in rate of oxygen consumption the tissue respiration lowers but slowly for several hours, 5 in this experiment, and 7 in another that gave very similar results in every way.[7]

Not only in the above data but in others to be presented it will be noticed that there is a rather large variation in check experiments. This variability may be due to a combination of factors. Very likely the 3 gm. portions used did not contain the same number of pieces of brain of the same size; in another experiment it was shown that while this factor did not make a great difference, the difference was appreciable. Another factor entering into the above variations is the thoroughness with which the water present was mixed with the tissue. Though every tube was treated as nearly as possible alike, there were surely small differences. The weight of the tissue used (3 gm.) varied considerably. This was probably a large factor in producing the variations in the above table. The tissue was weighed on small horn pan balances. It was necessary to weigh quickly to avoid effect of air, and especially to avoid the effect of standing on the rate of respiration.

Comparison of Brain Respiration with That of Other Tissues.—For the sake of comparison, and because so much more work has

been done on other tissues, the following experiments were carried out on brain, liver, kidney, and muscle. 3 gm. portions of these organs from guinea pigs were run in as nearly as possible the same way at the same time, to show what were the differences in oxygen consumption and carbon dioxide production.

TABLE VI.

Time in hrs.	Hg. by manometer readings. ½	1	2	3	Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
Old guinea pig. O reading 40 min. after death.								
	mm.	*mm.*	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
Brain	13	17	30	44	0.88	3.6	0.074	0.076
Liver	10	20	42	62	1.24	3.8	0.118	0.080
Kidney	8	14	28	44	0.88	3.4	0.074	0.071
Muscle	6	10	20	31	0.62	2.7	0.059	0.057
Younger guinea pig. O reading 1 hr. after death.								
Brain	12	20	50	72	1.44	4.4	0.137	0.093
Liver	13	24	58	78	1.56	4.6	0.148	0.097
Muscle	8	14	26	42	0.84	2.6	0.080	0.055
Old guinea pig. O reading 40 min. after death.								
Brain	9	16	32	44	0.88	4.1	0.084	0.086
Liver	15	26	48	64	1.28	4.9	0.122	0.103
Kidney	10	20	40	52	1.04	4.1	0.099	0.086
Muscle	7	10	20	32	0.64	3.1	0.061	0.065

The rate of oxidation of ground brain tissue 1 to 3 hours after death is not quite so great as that of liver from the same animal treated in the same way. It has about the same rate as the kidney and is considerably larger than that of the muscles. If there is a primary respiration occurring in the living intact cells that rapidly disappears in the treatment used in these experiments, the data given furnish a comparison of the secondary respiration only.

It will be noticed that the oxidation in brain tissue does not increase as rapidly during the 2nd and 3rd hours as does that for the other tissues. May this be connected with the fact that autolysis of brain is so much slower than that of other tissues? There seems to be more protective material to keep the brain in its original condition than in other tissues.

Divisions of the Nervous System.—The question that was most carefully worked out was the comparison of the respiration of the various parts of the brain. Samples from various parts of the brain of a single animal were treated in exactly the same way and were run at the same time.

TABLE VII.

Time in hrs.	Hg. by manometer readings. ½	1	2	3	Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
6 mos. old calf. O reading 1 hr. 50 min. after death.								
	mm.	*mm.*	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
Cerebrum	8	16	40	65	1.30	4.1	0.123	0.086
Cerebellum	9	17	45	70	1.40	4.1	0.133	0.086
Midbrain	7	16	38	60	1.20	4.0	0.114	0.084
Medulla	8	12	32	50	1.00	3.6	0.095	0.076
Corpus callosum	5	10	30	45	0.90	3.6	0.086	0.076
Spinal cord	5	10	26	45	0.90	3.4	0.086	0.071
Sciatic nerve	5	10	25	42	0.84	4.7	0.080	0.099
Old beef. O reading 2 hrs. 15 min. after death.								
Cerebrum	7	15	30	45	0.90	3.3	0.086	0.069
	8	14	26	40	0.80	3.4	0.076	0.071
Cerebellum	11	17	32	46	0.92	3.1	0.087	0.065
	10	15	28	44	0.88	3.1	0.084	0.065
Midbrain	6	13	21	30	0.60	3.3	0.057	0.069
	7	15	24	36	0.72	3.3	0.068	0.069
Corpus callosum	5	9	15	25	0.50	2.7	0.048	0.057
	6	11	18	27	0.54	2.7	0.052	0.057
Spinal cord	4	6	12	17	0.34	2.1	0.032	0.044
	5	7	12	16	0.32	2.0	0.030	0.044
1 yr. old calf. O reading 1 hr. 50 min. after death.								
Cerebrum	10	18	40	70	1.40	4.5	0.133	0.095
	10	19	38	68	1.36	4.4	0.129	0.092
Cerebellum	10	17	40	66	1.32	4.6	0.125	0.097
	9	16	37	67	1.34	4.4	0.127	0.092
Midbrain	5	10	24	46	0.92	3.0	0.087	0.063
	5	11	25	47	0.94	3.2	0.089	0.067
Medulla	5	10	20	32	0.64	2.8	0.061	0.059
	5	10	21	33	0.66	2.7	0.063	0.057
Corpus callosum	7	14	28	50	1.00	3.2	0.095	0.067
	8	15	32	48	0.96	3.1	0.091	0.065
Spinal cord	4	7	15	26	0.52	2.7	0.050	0.057
	4	8	17	28	0.56	2.7	0.054	0.057

There is but little difference either in oxygen consumption or carbon dioxide production between the cerebrum and the cerebellum. The midbrain respires more slowly than these but not so slowly as the medulla, whose rate is about three-fourths of that of the cerebellum. Both the oxygen used and the carbon dioxide formed are still less in the corpus callosum, but the carbon dioxide is not generally as much less as is the oxygen. It will be noticed throughout these results that the more medullary sheath material there is present the higher the relative carbon dioxide production. This may indicate that a part at least of the respiration in nerve fiber is different from that in nerve cell. This is very apparent in the spinal cord, where the oxygen consumed is about half of that in the cerebrum, while the carbon dioxide produced is about two-thirds as much as in the cerebrum. Though there is but one experiment in which sciatic nerve is compared with the other divisions of the nervous system, the rate of oxygen consumption is even less than that of the spinal cord; but the carbon dioxide produced is even larger than that in the cerebrum. This may be an error, but is very interesting if true. This gives a respiratory quotient much larger than for the other portions of the nervous system.[9]

It would, of course, be premature to attempt to correlate in any way the data given with the degree of differentiation of the different brain divisions or with their biological functions.

Gray and White Matter.—Gray matter selected from the outside

TABLE VIII.

Calf Brain. O Reading 1½ Hrs. after Death.

Time in hrs.	Hg. by manometer readings.			Total O consumed	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
	1	2	3				
	mm.	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
Gray	25	45	65	0.30	4.0	0.124	0.084
	24	42	62	0.24	4.2	0.118	0.088
	24	44	60	0.20	4.1	0.114	0.086
White	14	26	35	0.70	2.6	0.067	0.055
	15	25	34	0.68	2.8	0.065	0.059
	12	24	35	0.70	3.0	0.067	0.063

[9] Tashiro, *Am. J. Physiol.*, 1913, xxxii, 139.

layer of the cortex was compared with white matter from the corpus callosum.

As was to be expected from the preceding tables, gray matter uses more oxygen and forms more carbon dioxide than white matter. There is almost twice as much oxygen used by the gray, but the white produces about two-thirds as much carbon dioxide as does the gray. The respiratory quotient is consequently higher in white matter than in gray. This table also emphasizes the fact that there is a higher respiratory quotient where the amount of medullation is greater.

Work now in progress on the autorespiration of cephalin, which occurs in much greater amount in white matter, may furnish a clue to this difference between white and gray matter. There is considerable indirect evidence also that the nucleoproteins are concerned with brain respiration.[10] It would simplify matters greatly if it were found that a lipoid was concerned with one form of respiration and a nucleoprotein with another.

Comparison of Ages of Animals.—It is to be expected that the younger the brain, the faster its metabolism. We know this to be true for the whole animal. This is supposed to be due to growth of cells, and a larger proportion of the more reactive sub-

TABLE IX.

Time in hrs.	Hg. by manometer readings. ½	1	2	3	Total O consumed	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
Beef.								
	mm.	*mm.*	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
Cerebrum, 8 mos.	11	20	45	62	1.24	4.1	0.118	0.086
" old	7	15	30	45	0.90	3.2	0.086	0.067
Cerebellum, 8 mos.	10	20	45	60	1.20	4.0	0.114	0.084
" old	10	15	28	44	0.88	3.1	0.084	0.065
Dog.								
Whole brain, 3 mos.	13	23	40	57	1.14	3.8	0.108	0.080
" " fat and old.	10	18	30	42	0.84	2.9	0.080	0.061
" " adult	9	16	28	38	0.76	3.2	0.074	0.067

[10] McGregor, H. H., *J. Biol. Chem.*, 1916–17, xxviii, 403. Batelli and Stern, *Biochem. Z.*, 1911, xxxiv, 263. Lillie, R. S., *Am. J. Physiol.*, 1902, vii, 412.

stances in the cells, with a consequent smaller percentage of the less reactive colloids.[11]

By consulting previous tables it will be seen that in each division of the brain the same decrease in rate of respiration occurs. What is true of respiration is undoubtedly true of its complement metabolism. With an increase in amount of oxidation, there must be an increase in amount of food substances oxidized, with a corresponding increase in metabolic products excreted. During growth the rate of oxidation decreases, but the complexity of the reaction brought about by the oxidation increases.

Comparison of Different Adult Animals.—Though the animals used in these experiments were adults they varied greatly in size, age, and nutritional condition. The figures in parentheses in the first column (Table X) give the number of experiments averaged to give the results in the table. The table is arranged in order of size of the animals.

TABLE X.

O Reading about 2 Hrs. after Death.

Time in hrs	Hg. by manometer readings.				Total O consumed.	0.02 N alkali used by CO_2.	O per gm. per hr.	CO_2 per gm. per hr.
	½	1	2	3				
	mm.	*mm.*	*mm.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*
(3) Frog, brain and spinal cord	5	10	18	32	0.64	1.8	0.061	0.038
(3) Mouse	18	26	47	68	1.36	4.6	0.129	0.097
(2) Rat	10	21	38	57	1.14	4.4	0.108	0.092
(3) Guinea pig	10	18	35	58	1.16	4.3	0.110	0.090
(1) Cat	10	17	36	56	1.12	3.8	0.106	0.080
(2) Dog	9	17	32	44	0.88	3.3	0.084	0.069
(2) Sheep	8	15	28	48	0.96	3.6	0.091	0.076
(3) Pig	7	13	20	36	0.72	3.0	0.068	0.063
(3) Beef	7	15	30	45	0.90	3.2	0.086	0.067

The theory that the greater the surface area of an animal the greater will be its food and oxygen requirements is found to apply in a general way to brain respiration. In the brain, however, one would not expect so direct a relation between surface evaporation and rate of metabolism as in other parts of the body. It is interesting to note that the indolphenol oxidase content[12] of the

[11] Koch, W., and Koch, M. L., *J. Biol. Chem.*, 1913, xv, 423.

[12] Vernon, H. M., *J. Physiol.*, 1911–12, xliii, 96.

brains of animals varies with size in a similar way to that of respiration.

The frog brain and spinal cord do not seem to have as great a capacity for oxidation as those of the higher animals. On first thought one might expect that with evolution there would be greater oxidative efficiency. But a consideration of the data for mammals (though but suggestive because of lack of higher forms) indicates brain size as another factor in mental complexity. If rate of respiration were a measure of brain ability, the mouse should be capable of more complex activity than the dog or sheep; but because the brain of the dog or sheep is larger (thus having more nerve cells and more connections) their mental power, as well as their total brain oxidation, is greater. There is also reason for believing that the nature and amounts of the colloidal substances in nerve cells are important in determining the intensity and character of the response. Judging from other data as well as from those given in this paper, brain evolution, from both the phylogenetic and ontogenetic standpoints, seems to be accompanied by a decrease in the rate of chemical activity, but by an increase in the complexity of the colloidal substratum and an increase in the number of cells.

SUMMARY.

1. The order in which the various divisions of the nervous system respire was found to be the following: cerebrum, cerebellum, midbrain, medulla, corpus callosum, spinal cord, then nerve.

2. The smaller the animal the more rapidly, as a rule, its brain consumes oxygen and gives off carbon dioxide.

3. In an adult animal brain respiration is less than in a young one.

4. Gray matter consumes about twice as much oxygen as white matter, but about two-thirds as much carbon dioxide is produced by the latter.

5. Grinding brain tissue with sand destroys its power of autoxidation.

6. Heating to 100° or addition of toluene (probably anesthetics too) reduces the amount of respiration more than half, indicating a thermostabile and thermolabile factor.

7. The temperature coefficient for brain oxidation is about 1.8.

8. Ground guinea pig brain tissue oxidizes at about the same rate as kidney tissue, faster than muscle, but not so rapidly as liver. There is not, however, a large difference between them.

9. Blood in brain tissue may have a small catalyzing effect in very small quantities, but when appreciable amounts are present, its respiratory effect is but little more than additive.

THE DIRECT AND INDIRECT CALORIMETRY OF CASSIOPEA XAMACHANA.

THE EFFECT OF STRETCHING ON THE RATE OF THE NERVE IMPULSE.

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The object of the present paper is to throw light on the factors influencing the oxidation rate of living cells. Henze (1910, *a*) observed that sea anemones use less oxygen when less is present in the sea water, but interpreted this as due to the time required for diffusion into the animal. That is to say, he supposed that all of the cells were not supplied with oxygen when there was little in the sea water. If oxygen was entirely absent in some of the cells, the decreased oxidation may have been merely the expression of the lesser number of cells taking part in the metabolism. Burrows showed that tissue cells require a certain oxygen tension for growth, and Loeb and Wasteneys found that the heart beat of *Fundulus* embryos may be slowed by reducing the oxygen. The growth of *Fundulus* may be suspended by lack of oxygen and may be slowed by diminishing the oxygen.

EXPERIMENTAL.

The calorimeter consisted of either an 840 cc. or a 900 cc. Dewar flask (thermos bottle) enclosed in an air tight container, which was immersed in water that was maintained at the same temperature as the water in the flask, within 0.003°. The technical difficulties were met as follows: Two Beckmann thermometers were adjusted and compared over the range of temperatures of the experiments, and fitted with reading lenses to estimate down to 0.001°. A large tank of sea water was brought to 30° (which was about the temperature of the air) and its pH and O_2 and CO_2 content determined. Some of this sea water was dipped out and a *Cassiopea* in-

troduced into it. The thermos bottle, stopper, and thermometers were immersed in the large tank until they reached the temperature of the water. The *Cassiopea* was transferred to the thermos bottle and a perforated cork stopper inserted with the exclusion of air bubbles. One Beckmann thermometer was inserted through the perforation in the stopper and the other suspended in the tank, near the middle of the thermos bottle. A small hole, remaining in the stopper for exit of displaced water, was closed with wax. The pulsations of the *Cassiopea* stirred the water inside the thermos bottle; and the water in the tank was mechanically stirred and was kept at the same temperature as that inside the thermos bottle by additions of small portions of warmer or colder water as required. The light was excluded by the silvering and coverings of the thermos bottle, but in some experiments, in which a 900 cc. glass jar with ground glass cover was used in place of the thermos bottle, the light could be excluded by darkening the tank, so as to prevent photosynthesis in the symbiotic plant cells. Time was measured by means of a stop-watch and a clock. The same *Cassiopea* was used in a series of experiments.

The oxygen in the sea water was determined by the Winkler method, which can be corrected for the slight error due to a slight amount of organic matter given out by *Cassiopea*, and it was thought impracticable to use the complicated method of Shutzenberger and Risler (Henze, 1910, *b*). A 250 cc. glass-stoppered bottle was weighed empty and full of distilled water at a known temperature, in order to standardize its volume. It was fitted with a double-bored rubber stopper and a long and a short glass tube with rubber connections. The bottle was filled with mercury and the long glass tube sucked full of the water to be analyzed and the stopper inserted. By inverting the bottle, the sea water was siphoned into it, when the rubber stopper was removed and the glass stopper inserted. The glass stopper was lifted and 1 cc. of alkaline KI solution and 1 cc. of 40 per cent $MnCl_2$ solution introduced (correction being made for this in the O_2 calculation) and the stopper inserted. Colloidal membranes formed about the drops of alkali and violent shaking was necessary to break them. After the precipitate had settled, 2 cc. of concentrated HCl were added and the stopper inserted and the bottle shaken. Its contents were transferred to a flask and titrated with 0.01 N sodium thiosulfate solution until the yellow color disappeared, then starch solution was added and the titration continued until the blue color disappeared, then the water was poured into the bottle and back into the flask and titrated until the blue color disappeared. The calculation was as follows: $\frac{0.056 \times \text{cc. thiosulfate}}{\text{capacity of bottle} - 2}$ = cc. of oxygen per liter of sea water, and this quotient $\times$ 0.9 = cc. of oxygen in calorimeter (on the assumption that the concentration of oxygen in the *Cassiopea* was the same as that in the sea water).

In order to reduce the carbonate content, the alkaline KI solution was made fresh every few days from two stock solutions. Carbonate-free NaOH solution was made by dissolving 100 gm. of NaOH in 100 cc. H_2O

in a glass-stoppered bottle and pipetting off after the carbonate had settled. One part of this was mixed with one part of 20 per cent KI solution before being used. NaOH made from metallic sodium contained a trace of nitrite and NaOH purified by alcohol contained but little more. This nitrite causes no error if the titration is quickly made immediately after adding the acid, but the contents of the flask slowly turn blue for hours after the end-point has been reached. If the acidity is greatly increased, however, the nitrite causes an appreciable error in the titration.

The $MnCl_2$ solution contained a trace of $Mn(OH)_3$, but this was removed by decantation. The thiosulfate was dissolved in CO_2-free water and kept in an automatic burette with soda-lime tubes, and standardized with pure iodine. The starch solution was allowed to settle and only the clear upper portion used. When dextrins appeared in it, a fresh solution was prepared.

The number of gm. of chlorine per kilo of sea water (abbreviated Cl) was determined by titrating with a silver nitrate solution standardized with standard sea water from the International Commission. The alkaline reserve was determined by titrating 100 cc. of sea water with 0.01 N HCl, while boiling in a 500 cc. Erlenmeyer flask of resistance glass, using dibrom-*o*-cresolsulfophthalein as indicator, until every trace of purple color had permanently disappeared. The water must not dry on the sides of the flask. The pH was determined colorimetrically by means of the method and identical tubes previously described (McClendon, 1917, *b*). Correction was made for Cl. It was found that new supplies of indicators must be tested before being used to see that they have the proper pH range. The total CO_2 was determined from the pH and alkaline reserve by means of the conversion table (McClendon, 1917, *b*).

The weights and one thermometer were standardized by the United States Bureau of Standards and the other apparatus was standardized with them.

Although pH and CO_2 determinations were made in all experiments, it was found that the oxygen absorbed could be determined much more accurately than the CO_2 given out, and the CO_2 determinations are not listed with the experiments, but are collected together in the form of respiratory quotients. The respiratory quotients were 0.7, 0.74, 0.76, 0.84, 0.85, 0.86, 0.88, 0.9, 0.91, 0.92, 0.97, 0.99, 1, 1.02, 1.03, 1.1, 1.15, 1.2. It was impossible to tell whether this variation is due entirely to errors in the CO_2 determinations or whether the respiratory quotient varied. It is improbable, however, that respiratory quotients of 1.2 existed for even short periods of time, and these at least may be considered due to technical errors. According to Mayer (1914) *Cassiopea* lives on animal food exclusively and does not

absorb carbohydrates from its symbiotic algæ, since it starves as rapidly in the light as in the dark. It may, however, get some carbohydrates from its animal food, or from glycoproteins during starvation. In this connection it may be of interest to note that *Cassiopea* secreted a mucin-like substance. Since the error in estimating CO_2 production may be 30 per cent in half hour experiments with small *Cassiopeas*, it is convenient to assume that the respiratory quotient is constant and is about 0.95, which is also the average found by Vernon for the hardier species of jellyfish on which he made most of his determinations.

The oxygen consumption is about doubled when the temperature is raised from 20° to 30°, and from results on other animals is probably an exponential function of the difference in temperature. Harvey (Mayer, 1917) found the velocity of the nerve impulse in *Cassiopea* to be a linear function of the temperature, and to increase about 64 per cent on raising the temperature from 23° to 33°. I found the activity of the ganglia (rhopalia) in inducing pulsations of the umbrella to be about doubled with a rise from 20° to 30° in temperature. The data were as follows:

Cc. O_2 consumption.		Temperature coefficient for 10° rise.	Pulsations per sec.	
20°	30°		20°	30°
0.698	1.54	2.21		
0.57	1.05	1.84	0.27	0.55

These experiments show the necessity of accurate temperature control and in all of the other experiments in this paper the temperature was measured to the nearest tenth of a degree and maintained to within 0.2° of 30°.

The sea surface is at about the optimum pH for metabolism (usually 8.1–8.3), but the variation in metabolism with variation of pH within the range studied is very slight, as shown by the following table (diameter of *Cassiopea* = 10 cm.).

pH	O_2 concentration.	O_2 used.
7.50	4.40	1.85
7.53	4.50	1.95
8.24	4.30	2.79
8.24	4.50	2.87
8.24	4.55	2.43
8.38	4.40	2.07
8.52	3.90	2.28
8.72	3.16	2.59

These variations in oxidation may be due to experimental errors and variation in O_2 concentration, except the first two which show a slight lowering of oxidation when the pH is reduced to about 7.5.

In comparing the rise in temperature in the calorimeter with that calculated from the O_2 consumption, the assumptions were made that the respiratory quotient was 0.95, and that a mixture of proteins, fats, and carbohydrates was burned, giving 6 gm. calories per cc. CO_2, as in the following table.

O_2	CO_2	Gm. calories.	
		Determined.	Calculated.
2.18	2.07	12.6	12.4
2.54	2.41	16.2	14.5

In these experiments it was assumed that there was no loss of heat, although some heat must have passed into the thermometer bulb and glass lining of the thermos bottle. The specific heat of the sea water and *Cassiopea* was taken as unity because the determinations were not accurate enough to warrant the application of small corrections. The experiments had to continue for 2 hours in order to obtain an accurately measurable rise in temperature, and the tedium of keeping the tank at the same temperature as the calorimeter necessitated the substitution of indirect calorimetry in the remainder of the experiments.

Some preliminary experiments to show the effect of O_2 concentration are as follows:

Average O_2 concentration.....	11.9	1.91	3.36	1.55
O_2 consumption.....	2.6	2.38	2.26	1.65

In performing these experiments, a number of possible sources of error were thought of and it was decided to make a more detailed study of the metabolism of *Cassiopea* before returning to the subject. The chief danger of error was in prolonging the experiment until all of the O_2 was used up. It was found that *Cassiopea* could live more than 7 hours without oxygen, in which case no measurable quantity of CO_2 was produced. Vernon observed practically no increase in the respiratory quotient of jellyfish correlated with oxygen-want, whereas the respiratory quotient of fishes increased under these conditions.

In order to determine whether the rate of oxidation depends on the oxygen concentration it is desirable to know something about the oxygen concentration inside the living cells. In other words, the transfer of oxygen to the cells must be facilitated as much as possible if we are to judge anything about the concentration of O_2 within them from that in the sea water. This could be approximated by agitating free cells or a single layer of cells with the water or circulating the water over a single layer of cells. When using free cells, some are liable to injury and more or less disintegration, thus interfering with the titrations, but notwithstanding the criticism of Heilbrunn, comparative results may be obtained (McClendon and Mitchell). *Cassiopea* was chosen, because the cells are spread in thin layers on the surface of a mesoglea which will be shown to use practically no oxygen. The pulsations of the *Cassiopea* (Mayer, 1906) bring currents of water over the cell layers, so that diffusion is necessary only for a minute distance. The error due to this diffusion would be large only when the O_2 concentration is very small. By skilful manipulation, the mucous secretion may be prevented from increasing or leaving the surface of the *Cassiopea*.

Evidence that oxidation is confined to the cell layers is apparent in the fact that oxidation is not proportional to the volume but to the surface. It would be practically impossible to measure the surface, but since the individuals are practically of the same shape, the surface is proportional to the square of

the diameter. Since *Cassiopea* is elastic, the diameter was always measured under the same conditions; *i.e.*, resting on a glass plate, with the exumbrella in contact with the glass (and the average of two diameters at right angles to one another taken). Some rough preliminary determinations showed the O_2 consumption in cc. per hour to be about 0.023 × the square of the diameter in cc. as shown in the following table.

Diameter in cm.	3.5	7	8.5	9.5	10	11.5
O_2 per hr.	0.4	1.4	1.17	2.16	2.7	2.78

Very small *Cassiopeas* used more O_2 than calculated from the formula (an anomaly which is correlated with more rapid pulsations). A *Cassiopea* 3.5 cm. in diameter pulsated once a second whereas one 10 cm. in diameter pulsated 0.3 times per second. In order to compare experiments on *Cassiopea* where the weight is recorded, it is convenient to know that the diameter in cm. $= 2.25 \times \sqrt[3]{}$ of the weight in gm.

The following table gives the respiration rate under different conditions, except that the temperature is always 30°.

Diameter.	O_2 per hr. / square of diameter	O_2 per liter.	pH	O_2 per hr.
cm.				
7	0.0200	4.5	8.20	1.3
8	0.0187	4.2	8.22	1.26
8.5	0.0152	3.32	8.22	1.09
10	0.0287	4.5	8.24	2.87
10	0.0279	4.3	8.24	2.79
10	0.0243	4.55	8.24	2.43
10	0.0185	4.4	7.50	1.85
10	0.0195	4.5	7.53	1.95
10	0.0207	4.4	8.38	2.07
10	0.0228	3.9	8.52	2.28
10	0.0259	3.16	8.72	2.59
11	0.0230	3.54	8.17	2.79
11	0.0200	1.8	8.40	2.43
11	0.0160	1.57	8.02	1.93
11	0.0320	7.2	8.47	3.84
11	0.0400	7.44	8.46	4.87

In the above table the pH and O_2 per liter at the beginning of the experiment are given and the pH was about 0.09 and O_2 1.5 cc. lower at the end of each experiment. The average O_2 during each experiment influences the O_2 used per hour, but apparently no difference in the quotient of the O_2 used per hour divided by the square of the diameter (second column) can be correlated with difference in size. Using greater extremes of size, however, the quotient seems to decrease as the diameter increases, and therefore extreme sizes were usually avoided.

All of the experiments were made under conditions of starvation and hence the *Cassiopea* used its own substance as a source of energy. Starvation can hardly be considered a pathological process in *Cassiopea*, however, since it may remain alive for months without food, constantly decreasing in weight until it almost disappears before death. Mayer (1914) determined the loss in weight as about 5.6 per cent per day at about 30°, although no thermostat was used. If y is the weight at any moment and w is the weight when starvation commenced and n is the number of days of starvation,

$$y = w\ (1 - 0.056)^n$$

Since I found the diameter to be 2.25 times the cube root of the weight, if the weight were 100 gm., the diameter would be 10.45 cm. The O_2 consumed during 1 day would be about 0.023 times the square of the diameter times 24 = about 60 cc. O_2 absorbed and 57 cc. CO_2 given out. If we assume that protein was burned and that 5.9 gm. calories correspond to 1 cc. CO_2, the metabolism would equal 336 gm. calories for the day. If we assume a certain mixture of proteins, fats, and carbohydrates were burned and 6 calories correspond to 1 cc. CO_2, the metabolism would equal 342 calories per day.

Since I have shown that the metabolism is proportional to the surface and Mayer has shown that the loss in weight is proportional to the volume (weight), the composition of the *Cassiopea* must change during starvation. In other words, it loses weight faster than it burns protein (or other organic matter), and hence the concentration of the protein must increase. Mayer (1914) found the cellular layer did not decrease in thickness during starvation, and Hatai found the percentage of ni-

trogen to the total body weight increases during starvation, and is also greater in small than in large, well nourished *Cassiopeas*. Therefore in attempting to calculate the metabolism from the loss in body substance, we should make it proportional to the loss in surface rather than loss in volume, since the loss in living matter seems to be proportional to the loss in surface, and the *Cassiopea* seems to have no other important store of food than its own protoplasm, the mesoglea apparently functioning chiefly as a skeleton. Since the surface is proportional to the two-thirds power of the volume, we may assume that the protein is proportional to the two-thirds power of the weight (the density remaining practically constant). The protein equals 5.16 per cent of the two-thirds power of the weight (calculated from Hatai's data on the assumption that protein is 16 per cent N). The weight at the beginning of starvation was 100 gm. and the protein 1.107 gm.; at the end of 1 day the weight was 94.4 and the protein 1.07, being a loss of 37 mg. of protein. If we assume that 1 mg. of protein is equivalent to 4.4 calories the metabolism the 1st day would be 163 calories, although I found it to be 336–342 calories. Although these calculations are only approximate, since starvation is a little greater the 1st day than calculated by the formula, this great difference indicates that the burning of protein does not account for all of the heat. Since living cells contain lipoids or lipo-proteins, and carbohydrates or glyco-proteins, it seems probable that proteins, carbohydrates, and lipoids are burned. The mesoglea has not been analyzed separately, but is largely sea water, with possibly a trace of glyco-protein. It probably has little calorific value, since the use of a store of food would cause a relatively greater metabolism in large starving *Cassiopeas* than was actually observed.

Since the lining of the alimentary tract is not at the surface of the *Cassiopea* and O_2 must diffuse through at least a mm. of tissue to get to it, it was decided to pull off the manubrium and study the metabolism of the umbrella. The umbrella is disc-shaped and covered on both sides by epithelium, and pulsates, thus circulating the water. The wound made by removal of the manubrium is of small area and is covered by an epithelium within a few hours, and the umbrella will live as long as a starving *Cassiopea*. Some rough determinations indicate

that the respiration of the umbrella is only about a fourth as great as that of the whole *Cassiopea*. In the following table are recorded measurements on three *Cassiopeas* and on their umbrellas after removal.

Diameter.	O_2 per hr. / square of diameter		Pulsations per sec.
	Cassiopea.	Umbrella.	
cm.			
3.5	0.033	0.0075	0.94
9.5	0.021	0.0041	0.58
11.5	0.030	0.0075	0.63

Since the respiration is influenced by the muscular activity or pulsation rate and the latter is not constant, it was decided to remove the ganglia (rhopalia) that induce the pulsations and start a continuous contraction wave running around the subumbrella, the middle third of which has no neuromuscular tissue (Mayer, 1908). The rhopalia were cut out by means of a cork borer and the wave was started by electrical stimulation. It was noticed, however, that the contraction wave, apparently constant for short intervals of time changed more rapidly at first and then more slowly, but never became absolutely constant, the change being perhaps associated with shrinkage of the umbrella. The effect of shortening and stretching on the contraction wave was therefore studied. The rate of the contraction wave depends on the rate of the nerve impulse around the circuit of the nerve-muscle layer, but does not depend solely on the rate in the neuraxon, since there are numerous synapses, and furthermore, the path of the impulse is zigzag. Professor Cary kindly showed me a stained preparation of the nervous network of the subumbrella. Concentric rings cut from the umbrella are capable of maintaining a trapped wave for some time, but if the ring is too narrow, the wave cannot be started, or soon ceases after being started. Trapped waves can be started in two or three concentric rings cut from the umbrella and the wave revolves about the inner ring more often per second than about the outer ring, but the revolutions per second are not in exact inverse proportion to the mean diameters of the rings, or to the diameters of the inside tracks or holes in the rings. One subumbrella, 11.5 cm. in diam-

eter, was cut into two rings and waves were trapped in them. The wave in the inner ring made 2.5 revolutions per second, and the wave in the outer ring made 2 revolutions per second.

The uncertainty as to the length of the pace-making circuit that the nerve impulse takes around the ring may be avoided by stretching the inner edge of the ring until it is of the same diameter as the outer, thus transforming the ring into a cylinder or belt. Such a ring can be stretched further and behaves in a strikingly reversible manner. Since Mayer (1917) has shown that the rate of nerve conduction in *Cassiopea* depends on temperature and electric conductivity of the sea water, it should be noted that all the experiments in this paper, unless otherwise stated, were done in sea water of 30° and Cl = 20. Mayer found a variation of only about 2.5 per cent over the range of pH = 5.6–8.26, and this variation includes experimental errors and changes due to unknown causes. In the present experiments the pH was about 8.2 unless otherwise stated. The only difficulty in estimating the rate of the contraction wave arose from the fact that the rate is 1 to 5 per cent faster in the ring that has just been stretched than in the ring that has just been relaxed, depending on the degree of recent stretching or relaxation. If, however, the circumference of the ring is allowed to remain constant for 5 minutes after each short step of stretching or relaxation, the rate will approximate a mean value. This behavior of the ring may be regarded as a form of hysteresis, since the number of revolutions per second of the contraction wave tend to remain constant immediately after stretching or relaxation. I do not see how this can be explained on the assumption that the stretching of the neuraxon is the only factor, and it is significant to note that Carlson records no such hysteresis in the stretched nerve of the slug, where synapses are less numerous or entirely absent. One explanation of the increased rate immediately after stretching might be the thinning of the plasma membrane of the neuraxon, the regeneration in thickness taking appreciable time. Another suggestion is made that the immediate effect of stretching is increase in length of the neuraxon, but that this may be proportionately less than the increase in length of the strip of tissue, since the nerve paths may be straightened, and that the apparent hysteresis is due to the possibility that after the nerve is stretched synapses gradually

open, due to the tension, until the nerve paths are proportionately as zigzagged as at the start. This supposition may similarly be applied to the fact that by prodding a *Cassiopea* (having a trapped wave) with a stick, the number of revolutions of the wave per second is reduced, although no apparent increase in length of the conducting path remains. The local stretching of the subumbrella with the stick might break some of the synapses. Such complicated suppositions are not very useful, however, since they are not easily tested. A more probable hypothesis is given below in connection with the amplitude of the contraction wave. The significant fact is that (ignoring the period of readjustment) the ring may be stretched until the circumference is increased 72 per cent with practically no change in rate (mm. per second), although in order to accomplish this the number of revolutions per second or passages of the wave through the same tissue may be reduced 49 per cent. This is analogous to the effect of stretching a metallic wire on the passage of an electric current through it, with the difference that the process is completely reversible in *Cassiopea* after an increase of 72 per cent in length due to stretching. The results on two rings are as follows:

Length of circumference.	Rate (mm. per sec.)
mm.	
223	372
263	413
283	430
286	379
306	390
326	399
346	410
366	414
386	407
406	403
426	391
446	377
466	368
486	360
506	352
526	342

If the rate of wave propagation is the same, we would expect in umbrellas of *Cassiopeas* of different sizes that the number of revolutions of the wave per second would be inversely proportional to the diameter. The diameter is measured before the wave is started, and there is a progressive shrinkage in the diameter, due to starvation and decrease in volume, tension of the regenerating tissue after removal of the rhopalia, and increased tonus of the musculature (transforming the disc into a cup shape). This decrease in the diameter is associated with increase in revolutions per second, but agitation decreases the revolutions per second. It is therefore necessary to make the determinations under the same conditions for strictly comparative results. The following determinations were rough, but serve to indicate the general features.

Diameters.	Revolutions of wave per sec.		
	Immediately.	After 24 hrs.	After prodding with a stick.
cm.			
9.75	2.38	2.50	
10.50	1.79	2.17	2.04
10.50	1.75	2.17	2.04
10.75	1.75	2.13	
11.00	2.38	2.08	
11.50	1.72	2.22	
11.50	1.79	2.22	
12.50	1.67	1.72	1.66
12.80	1.67	2.00	
13.50	1.61	1.67	1.40
13.50	1.61	1.79	1.52
14.00	1.67	1.79	

A comparison of these experiments with those on the actual velocity of the wave shows the circumference of the potential pace-making circuit to be about 1.8 times the diameter. If the contraction wave is stopped by pressure and after a rest of some minutes or hours started again, it is slower than just before stopping, but if it is started again as quickly as possible after stoppage, the rate is the same. This effect of a rest seems to be associated with nutrition or recovery from fatigue, since the amplitude of the contraction wave is greater in the rested

umbrella, although the number of revolutions per second is decreased. Whether the actual rate of propagation is changed would be difficult to determine. It seems evident that the wave of nerve impulse precedes the wave of muscular contraction. The contraction of the muscle must stretch the adjacent regions, and hence stretch the region through which the nerve impulse is passing, thus increasing the distance traveled in one revolution and decreasing the revolutions per second. When the amplitude of contraction is increased, the stretching of the nerves is increased and the revolutions per second are decreased, but whether this can account for the total decrease has not been determined. The speeding up of the revolutions per second after the trapped wave is started is at first more abrupt and later more gradual, and is associated with both decrease in amplitude of contraction and decrease in diameter of the umbrella, due to starvation and contraction of scar tissue.

Evidently a change in the number of revolutions per second of the trapped wave or the amplitude of the contractions would cause an error in the determination of the effect of O_2 concentration on metabolism, and in order to estimate the limits of such errors, the relative metabolism of the muscle and other tissues was studied. The umbrella of three *Cassiopeas*, *A*, *B*, and *C*, of the same size (diameter = 11.5 cm.) were used (each for a series of experiments). In some experiments the rhopalia remained and the normal pulsations were generated, in others a trapped wave was induced, and in others the subumbrella was removed or merely the mesoglea left. The apparent (but slight) metabolism of the mesoglea was probably entirely due to a few remnants of epithelium and to bacteria, which always attack the mesoglea when the epithelium is removed. At any rate the metabolism of the mesoglea is too small to be of significance. The pH was 8.2 and the O_2 per liter 4.5 cc. at the beginning of each of the experiments.

The removal of the rhopalia in the umbrellas with trapped waves or without pulsations, reduced but slightly the amount of tissue. If we take the metabolism of the normal pulsating umbrella as 100, the exumbrella is about 14 and the resting subumbrella 60 with an addition of 26 for normal pulsations or 65 for trapped wave. Therefore, the neuromuscular tissue may per-

Individual.	O_2 per hr.	Parts of umbrella used.
	cc.	
A	0.86	Umbrella with 0.64 pulsations per sec.
A	0.97	" " 0.64 " " "
A	0.158	Mesoglea + exumbrella only.
B	1.12	Umbrella with trapped wave, 1.47 per sec.
B	1.08	" " " " 1.47 " "
C	0.5	" not pulsating.
C	0.6	" " "
C	0.03	Mesoglea + bacteria.
D	0.88	Umbrella with 0.7 pulsations per second.
D	0.7	" not pulsating.

form about 26 to 46 per cent of the metabolism, and changes in rate or amplitude of the contraction wave are to be avoided as much as possible. In subsequent experiments, the manubrium and rhopalia were removed from the *Cassiopea*, and a C-shaped cut was made through the neuromuscular layer about one-third the radius from the outer margin of the umbrella, and a trapped wave induced by stimulation near the outer margin. The wave passed around the outer part of the subumbrella, and each time it passed the opening of the C it spread to the inner part, dividing into two equal waves, meeting on the far side with mutual destruction. In this way, the outer part, in which the trapped wave was first induced, was made pace-maker, and the result was a more permanent wave. The wave causes circulation of water against all parts of the epithelium except a small portion of the exumbrella which has a very low metabolism. If the oxygen concentration was reduced to zero, the wave stopped, and metabolism ceased until oxygen was readmitted. In one experiment an umbrella was kept 7 hours at zero O_2 concentration, then 3 hours with O_2 and a trapped wave, and 7 hours at zero O_2 concentration, during which it gave out no CO_2 or other acid products affecting the pH perceptibly. 30 seconds after it was taken out of the O_2-free chamber, a trapped wave was started and this constantly increased in amplitude for 10 minutes, at the end of which time the amplitude was normal. Oxygen was removed from sea water in various ways, with the air pump and agitation, by boiling, and by allowing a *Cassiopea* to remain

in it until the pulsations ceased, as seen through a peep hole in such a way that photosynthesis was practically avoided. In no case did the analysis show less than about 0.05 cc. per liter, but that amount probably entered with the KI and $MnCl_2$ solutions and around the ground stopper of the analysis bottle. At any rate, we should consider 0.05 cc. to be within the limit of error of the method if no correction be made for O_2 in the reagents.

The variation in metabolism after removal of the manubrium and initiation of a trapped wave is shown in the following table. The diameter of the umbrella was 11.5 cm. at the beginning of the first experiment and at the beginning of each experiment the pH was 8.2 and the O_2 per liter 4.5 cc.

Hrs. after operation.	O_2 used per hr.	Revolutions of wave per sec.
	cc.	
0	1.65	1.60
2	1.55	2.00
3	1.30	2.22
4	1.30	2.22
5	1.30	2.22
20	1.30	2.22

From the above table it is evident that the metabolic rate may vary rapidly for 3 hours after the manubrium is removed and the trapped wave is started, therefore in the subsequent experiments, the umbrella was not placed in the respiration chamber until these 3 hours had passed. The same umbrella used in the above experiments (but 21 hours after the operation) was used to determine the effect of reduced oxygen concentration. The average O_2 concentration during the experiment was 1.5 cc. per liter and the O_2 used per hour 1.03 cc., being a decline of 20 per cent in rate of oxidation with a reduction of the oxygen concentration to about half its original value. This and some later experiments are tabulated as follows:

At 3.3 cc. O_2 per liter. O_2 used per hr.	At 1.5 cc. O_2 per liter. O_2 used per hr.
1.3	1.03
1.3	1.00
1.4	1.10
1.48	1.15
1.44	1.25
1.35	1.05

These determinations show the oxidation is reduced about 20 per cent when the O_2 concentration is reduced about 50 per cent, or the oxidation increased about 25 per cent when the O_2 concentration is increased about 100 per cent.

DISCUSSION.

The above experiments show that the rate of oxidation varies with the concentration of oxygen in the sea water constantly circulated against the surface of the epithelium in which oxidation takes place. In the total absence of oxygen, no measurable quantity of CO_2 or other acid products is given out, and we may infer that the metabolism is suspended. If oxygen is readmitted after a suspension of the metabolism for 7 hours, the rate of metabolism rises apparently to the normal within 10 minutes. No anaerobic processes were detected during the absence of oxygen for 7 hours, but in the absence of oxygen for 16 hours, anaerobic or hydrolytic processes take place. No evidence was found to indicate that these anaerobic processes constituted the metabolism of the *Cassiopea*. On the contrary, a great multiplication of bacteria was associated with them, and the *Cassiopea* was partly dissolved and could not be revived by readmission of oxygen. We may assume that oxygen protects the *Cassiopea* from the attacks of bacteria (probably anaerobes). Since the bacteria enter from the surface, it seems probable that the *Cassiopea* might live indefinitely on so small a supply of oxygen that it is used in the superficial cells as fast as it diffuses into them and none reaches the deeper cells. If this be true, the variation in oxygen consumption with variation of supply might be the expression of a variation in the number of cells receiving oxygen. In other words, this would be a diffusion phenomenon.

Another hypothesis is that the concentration of oxygen at the seat of oxidation affects the rate. Warburg has shown that cell oxidation is associated with structure and that no enzyme solution has been obtained that will account for the vital oxidation of foodstuffs. If we call the structure or surface responsible for the oxidations the catalyst, it seems possible that the concentration of oxygen in the immediate vicinity of the catalyst influences the rate. This influence of concentration on rate might still be a diffusion phenomenon since the O_2 must diffuse toward the structure catalyst or oxidase molecule.

Owing to the excellent review of the literature on the subject of this paper by Krogh, it seems unnecessary to multiply references. Roughly speaking, and within physiological limits, animal oxidation is about doubled with 10° rise in temperature and this is shown here to be true of *Cassiopea*. In other words, oxidation is an exponential function of the temperature, as expressed in the following equation:

$$V_t = V_o \times 2^{\left(\frac{t}{10}\right)}$$

Where V_t is the velocity of oxidation at $t°$ and V_o at 0°. This relation holds approximately true for a number of chemical reactions, and is supposed to be due partly to change in diffusion rate and partly to loosening of bonds in the reacting molecules, and to ionization. Diffusion depends on viscosity and molecular motion. A fall of temperature from 30° to 20° increases the viscosity of water about 25 per cent, and of a 40 per cent sucrose solution 42 per cent and of a 3 per cent gelatin solution 1,000 per cent (von Schroeder). The increase in viscosity of gelatin, however, does not cause a proportionate decrease in diffusion. We may assume that this fall of temperature may possibly cause about 30 per cent decrease in diffusion of O_2 within the cell, due to viscosity alone. Molecular motion is proportional to the absolute temperature, and hence is a minor factor in diffusion. Although diffusion accounts for only about a third of the change in rate of oxidation with temperature, it is undoubtedly a factor. Since diffusion affects oxidation and concentration gradient affects diffusion, it seems very probable that concentration of O_2 should affect oxidation, even though every cell received some

oxygen. Krogh interprets the experiments on warm-blooded animals as showing increased oxidation with increased oxygen tension, and reviews the work of Thunberg, showing the same effect to a greater degree on cold-blooded animals. Henze (1910, *a*) found that the oxidation rate of sea anemones and annelids varies with O_2 concentration (and of nudibranchs at low concentrations) and supposed that only a varying fraction of the cells received oxygen. His tables show that the oxidation rate of crustacea and jellyfish fell rapidly with time, but that in one series of determinations on *Pelagia*, the oxidation rate varied reversibly with O_2 concentration. In order to reduce the diffusion effect, he kept sea urchin eggs agitated in sea water and found that the oxidation rate apparently increased about 8 per cent on doubling the O_2 concentration. Henze attributes this apparent difference in respiration to faulty technique, but since the experiments have not been repeated with improved technique, we may assume that a real difference exists.

It is interesting to compare the metabolism of *Cassiopea* with that of jellyfish studied by Vernon. Since only the living cells metabolize, it would be an advantage to know the proportion of cellular tissue to the body weight, but there is no data on this subject. The skeletal structure, mesoglea, contains less organic matter than the cells, and hence the per cent of organic matter in the body is a partial indication of the cellular mass. The proportion of mesoglea increases with the size of the individual within the same species, but there can be no strict comparison between different species in this regard, and therefore the comparison is very crude. The number of cc. of O_2 per hour per kilo of body weight and per kilo of organic matter (= dry weight − weight of salts in equal volume of sea water) at 20° is given in the following table.

Species.	Weight of individual.	Cc. O_2 per hr.	
		Per kilo of body weight.	Per kilo of organic matter.
	gm.		
Cassiopea xamachana	100	12.5	1,040
Carmarina hastata	30	7.7	2,025
Cestus veneris	70	3.75	1,562

The agreement is about as close as could be expected.

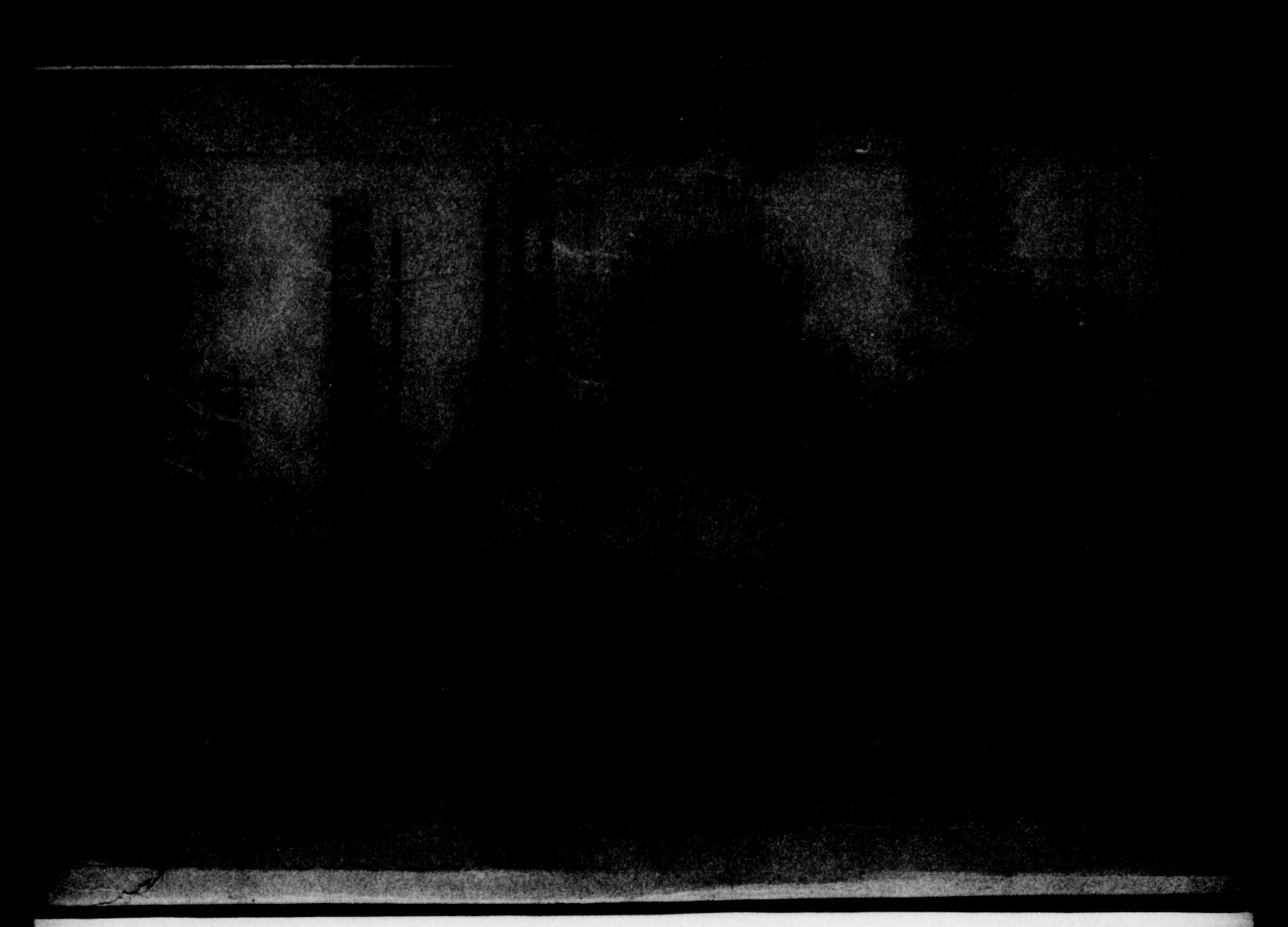
Vernon has compared the metabolism (per unit weight of organic matter) of jellyfish, molluses, tunicates, and vertebrates, and shown it to be remarkably constant. Krogh obtained greater differences when the total body weights were used (omitting jellyfish but including eggs and insects), but the differences probably do not exceed the differences in water content and in muscular activity. He found the metabolism of a young dog with body temperature reduced to 20° to be greater than that of cold-blooded animals at the same temperature, but if we reduce the metabolism of the average dog for 20°, using a reasonably high temperature coefficient, the agreement is more satisfactory. We should not expect close agreement unless water and mineral salts and fibrous tissue are excluded from the weight, and the activity of the nervous system is abolished. It seems probable, therefore, that the chief distinction in the calorimetry of warm-blooded and cold-blooded animals is in insulation. All warm-blooded animals are air breathing, and air brings much oxygen and takes away little heat. The center of a cluster of bees in winter may be 40° above that of the air bathing it.

The heat production in a 100 gm. *Cassiopea* at 30° is sufficient to raise its body temperature 0.14° per hour above that of the surrounding water, but no such difference in temperature has been observed because the heat generated is conducted away by the water bringing the oxygen. I found a fish weighing 1.4 gm. to use 0.825 cc. O_2 per hour at 30°, which is sufficient to raise its body temperature about 3° per hour, but during this time it was required to breathe 400 cc. of sea water, even though it removed half of the oxygen from water saturated with air at this temperature. The water circulating through the gills could remove the heat generated if the body temperature were 0.01° above that of the water. Since there was considerable conduction through the skin, and the fish probably removed much less than half the oxygen from the water in one passage through the gills, the body temperature was probably much less than 0.01° above that of the water. Rogers and Lewis could detect no difference between the body temperature of fish, salamanders, clams, and earthworms and the water in the thermostat in which they were placed, after they had been in the thermostat

long enough for equilibrium. They used a thermocouple, and one division of the galvanometer scale corresponded to 0.0042°.

It was shown that the metabolism of *Cassiopea* is proportional to its surface and not to its weight but to $W^{\frac{2}{3}}$. This is due to the fact that the metabolism is confined to the living cells and that these constitute a superficial epithelium, whose thickness is about the same in *Cassiopeas* of the range of sizes studied. We might use these results in an attempt to explain the so called surface law of warm-blooded animals. Dreyer, Ray, and Walker have shown that the blood volume and cross-sections of the aorta and trachea are proportional to $W^{\frac{2}{3}}$ (or surface). If animals are of the same shape (internally as well as externally) the cross-sections of all organs would be proportional to $W^{\frac{2}{3}}$ but the blood volume would be proportional to the weight (W). If the blood volume is proportional to $W^{\frac{2}{3}}$ the whole circulatory system would be nearly proportional to $W^{\frac{2}{3}}$ and owing to the close relation between the lungs and the blood, the volume of the lungs would be nearly proportional to $W^{\frac{2}{3}}$. The volume of the skin may be proportional to $W^{\frac{2}{3}}$ and the volume of the wall of the alimentary tract nearly so. The nerve, muscle, and glandular tissues are excitable, and hence their metabolism must vary. Variable components may be excluded from basal metabolism by definition but cannot all be excluded in making measurements. Only the skeleton can be said to have a metabolism proportional to W, and since the red bone marrow produces blood (erythrocytes) and this is proportional to the surface, the metabolism of some of the bones may be nearly proportional to the surface. Benedict has shown that great variations from the surface law exist, and hence it may be only accidental. The fact that the excitable tissues metabolize more per unit weight in small animals than in large (*i.e.*, proportional to a smaller power of W than unity) is true, not only for warm-blooded animals, but also for cold-blooded animals, to which the teleological principle of the surface law (in relation to heat regulation) does not apply. It seems possible, however, that the chief conditions necessary for the evolution of temperature-regulation in animals were: (1) air breathing, (2) large body size, (3) sensitivity to low temperatures, (4) variation of activity of excitable tissues with size, and

(5) epithelial type of architecture, (4) and (5) being common to cold-blooded animals.

BIBLIOGRAPHY.

For an extensive bibliography see Krogh and McClendon, 1917 (*a*).
Benedict, F. G., *J. Biol. Chem.*, 1915, xx, 263.
Burrows, M. T., *Am. J. Physiol.*, 1917, xliii, 13.
Carlson, A. J., *Am. J. Physiol.*, 1910-11, xxvii, 323.
Cary, L. R., *J. Exp. Zool.*, 1916, xxi, 1.
Dreyer, G., Ray, W., and Walker, E. W. A., *Proc. Roy. Soc., Series B*, 1912-13, lxxxvi, 39, 56.
Hatai, S., *Proc. Nat. Acad. Sc.*, 1917, iii, 22.
Heilbrunn, L. V., *Science*, 1915, xlii, 615.
Henze, M., *Biochem. Z.*, 1910 (*a*), xxvi, 266.
Henze, Abderhalden's Handb. biochem. Arbeitsmethoden, 1910 (*b*), iii, 1064.
Krogh, A., The Respiratory Exchange of Animals and Man, London, 1916.
Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1912, xl, 277.
Mayer, A. G., *Carnegie Institution of Washington, Publication No. 47*, 1906.
Mayer, *Carnegie Institution of Washington, Publication No. 102*, 1908, 115.
Mayer, *Carnegie Institution of Washington, Publication No. 183*, 1914, 55.
Mayer, *Am. J. Physiol.*, 1917, xlii, 469.
McClendon, J. F., Physical Chemistry of Vital Phenomena, Princeton, 1917 (*a*).
McClendon, *J. Biol. Chem.*, 1917 (*b*), xxx, 265.
McClendon, J. F., and Mitchell, P. H., *J. Biol. Chem.*, 1912, x, 459.
Rogers, C. G., and Lewis, E. M., *Biol. Bull.*, 1916, xxxi, 1.
Vernon, H. M., *J. Physiol.*, 1895, xix, 18.
von Schroeder, P., *Z. physik. Chem.*, 1903, xlv, 75.